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A Comparison of Impaired Function with Morphologic Changes Occurring in Livers of Rats Ingesting *N*-2-Fluorenyldiacetamide [1]

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A sensitive test of liver function has been proposed by Taplin, Meredith, and Kade (*1*). This test utilizes intravenously administered radioactive (I^{131} tagged) rose bengal, and provides a continuous *in vivo* record of uptake and clearance of radioactivity by the liver. Since the amount of radioactivity in the thyroid glands of rabbits 24 hours after intravenous injection of the tagged dye (*1*) was negligible, it was concluded that little, if any, I^{131} had been detached from the molecule. It seemed safe to assume, therefore, that the changes in radioactivity as measured over the liver indicated an in-and-out movement of the dye from this organ. Lowenstein (*2*) and Brown and Glasser (*3*), applying the test to man, concluded that it was of value in the differential diagnosis of diseases of the liver. According to Taplin *et al.* (*1*), the test also provided a direct measure of hepatic-cell function. The earlier observations of Mendeloff (*4*), who reported that fluorescence from rose bengal was found in hepatic cells only, following intravenous administration, also support the view that this dye gives a direct measure of hepatic-cell function.

A sensitive test that measured early changes in the ability of the liver cell to take up and release the dye was required in our studies, because it was desirable to identify the early stages in the development of neoplasms. It is well known that the reserve function of the liver is very great, and that many hepatic cells can be injured before functional impairment is detected. To cite two examples, McMaster and Rous (*5*), many years ago, found that 95 percent of the excretory function of the liver of the dog could be abolished before jaundice appeared, and Mann (*6*) found that the liver tissue in the dog could be reduced 80 percent or more without a decrease in urea production.

While the radioactive rose-bengal test was originally developed on the rabbit (*1*), it seemed feasible to use it on the rat. The rat liver is small, but the absence of a gall bladder appeared to be advantageous

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because in a small animal the dye that collects in a gall-bladder reservoir might interfere with measurements of radioactivity in hepatic cells.

Taplin *et al.* (*1*) found on histological examination of livers of rabbits damaged by chloroform that the degree of fatty degeneration of the hepatic cells roughly parallel the amplitude of the dye-uptake curve. These authors, however, did not mention other pathological alterations, such as cysts, basophilia, and central necrosis, that may occur in the livers of rats following administration of CCl_4 as described by Farber *et al.* (*7*). It is not known whether these alterations were present in the animals tested by Taplin *et al.* (*1*) and, if so, whether they contributed to the abnormal uptake-excretion pattern. The experiments described herein were designed to determine whether this new test of liver function would provide a means of detecting functional alterations which were associated with some of the morphologic changes known to take place during the development of carcinomas in rats ingesting the potent carcinogen *N*-2-fluorenyldiacetamide.

Materials and Methods

Strain A × C 9935 male rats weighing 140 to 220 gm. were divided into 2 groups, A and B. Group A consisted of 20 rats, 3 months old; 15 of these were fed an experimental diet (#284) containing the carcinogen *N*-2-fluorenyldiacetamide[5] and 5, used as controls, were fed the same diet minus the carcinogen (#285).[5] Group B comprised 55 rats 2½ to 3 months old; 40 were given diet #284 and 15, diet #285. Diet #284 [similar to diet #165 (*9*)] was used in this experiment because it has been found that many liver tumors develop rapidly on this regimen (*9*). The diet is very low in fat, moderately low in protein, and contains no added choline. Groups A and B were started on the diets 1 week apart. In group A the radioactive rose-bengal liver-function tests were made during the second, fourth, sixth, eighth, and tenth weeks of the experiment. Both control and treated rats of group A were given the function test at each of these biweekly intervals and all surviving at the end of 10 weeks were killed. Both control and treated rats of group B were divided into 4 subgroups at the start of the experiment. The rats of each of these subgroups were killed after periods of either 2, 4, 6, or 8 weeks. Each rat of group B, therefore, received the rose-bengal test once only, just before it was killed, and the liver was taken immediately for histologic study. Since acute effects, if any, were not likely to occur within 1 hour, the group B rats thus provided a control to determine whether the repeated injections of radioactive rose bengal given to the group A rats produced any injurious effects on the liver.

A few minutes before the liver-function test was started, each animal received an intraperitoneal injection of approximately 4.5 mg. sodium

[5] Diet #284 contained, in gm. per kg. of diet, the following constituents: vitamin-free casein 120; cerelose 746; salt mixture #185a 30 (*8*); coconut oil (containing 100 mg. α-tocopherol acetate and 3,000 units vitamin A acetate) 2.5, glycerol 100; linoleic acid 0.5; ascorbic acid 1, *N*-2-fluorenyldiacetamide 0.5; and a vitamin mixture containing per gm. of diet the following amounts of vitamin in μg.: folacin 1, biotin 0.01, thiamin hydrochloride 3, riboflavine 2, pyridoxine hydrochloride 2, calcium pantothenate 14, niacin 20.

Diet #285 was the same as diet #284, with *N*-2-fluorenyldiacetamide omitted.

pentobarbitol (Nembutal®) per 100 gm. of body weight. The hair was clipped from one hind leg, the abdomen, and the thorax. The rat was tied down on its back on a board. The lower borders of the ribs on the ventral surface were indicated roughly with a marking pen. By following these markings the animals could always be placed under the counter in the same position, and any variable because of the position of the liver was avoided.

The rat was placed under a DS-1 scintillation counter [6] with the xiphoid process centered directly under the opening of the collimated tube. The counter was connected through a power supply and a rate meter to an Esterline-Angus recorder.

The radioactive rose bengal (prepared by Abbott Laboratories) was given intravenously in a single 0.25 ml. dose. Both the concentration of the dye and the amount of radioactivity were found to be important in producing satisfactory tracings. Preliminary studies indicated that optimal curves for normal rats were obtained if the dose contained 8 μC I^{131} in 150 μg. of dye, with the counter scale set at 15,000 counts per minute. Greater amounts of the dye prevented the decline in the curve during the test period, probably because too much dye remained in the liver, thus reducing the sensitivity of the test. Smaller amounts of dye gave such a low maximal uptake that significant differences were not detectable. The proper concentration of dye was prepared by making appropriate dilutions of the radioactive dye with a 1 percent solution of nonradioactive rose bengal. The radioactive solution was injected into the saphenous vein after the rat was in position under the collimated tube of the counter, and a continuous record of the counts over the liver area was obtained for a 1-hour period.

The animals of group B were killed with ether within a few minutes after completion of the 1-hour radioactive rose-bengal test. The tissues were fixed in 10 percent neutral formalin and stained routinely with hematoxylin and eosin. Selected tissues were also treated with oil red O fat stain, Masson's trichrome stain, periodic-acid leucofuchsin Schiff reagent technique with diastase digestion, hematoxylin-eosin-azure stain, Wilder's silver impregnation for reticulum, Heidenhein's iron-hematoxylin stain, Clara's stain for bile canaliculi, phosphotungstic acid-hematoxylin stain mordanted with periodic acid, and Verhoeff's stain for acid-fast bacilli.

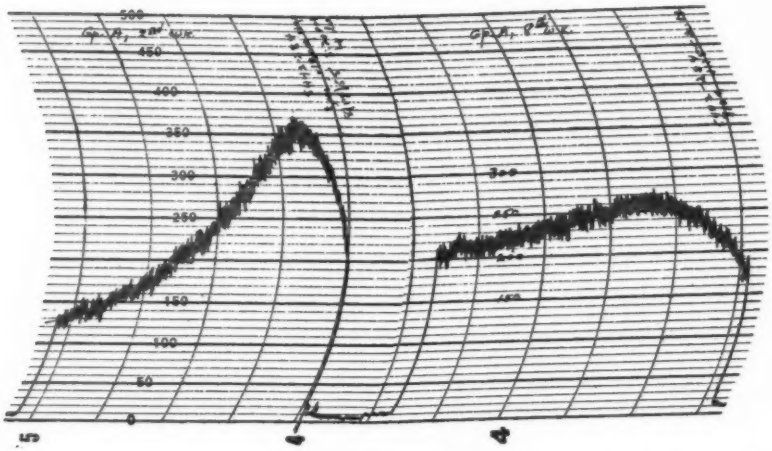
Results

The fluctuations in radioactivity following an injection of radioactive rose bengal are illustrated in text-figure 1. The ascending part of the tracing represents the flow of the radioactive material from the general circulation into the liver; the descending part represents its excretion from the liver primarily to the duodenum via the bile ducts. The uptake of the dye occurred in two phases: 1) A fast initial uptake phase, lasting only seconds, and 2) an uptake phase of decreased rate. In the

[6] We are indebted to Dr. John H. Weisburger for his assistance with the counting equipment.

carcinogen-fed animals beginning at 4 weeks the rate of uptake in this second phase was much slower than in controls and continued for a longer time before the maximum was reached (text-fig. 1, 8-week, *right*, *versus* 2-week, *left*, tracings). The functional test on an animal treated for 2 weeks, *left*, did not differ from that of a control.

The considerable fluctuation in the recorded counts, which occurred on every tracing, was smoothed by drawing freehand a continuous line approximating the mean counts along the tracing. Numerical values in terms of counts per minute were then obtained from the points where this freehand-drawn line intersected the time interval selected (usually every 5 minutes), beginning at injection time. The curves in text-figures 2 and 3 were plotted from the means of these values for each group of rats at each biweekly test period. These and subsequent curves were

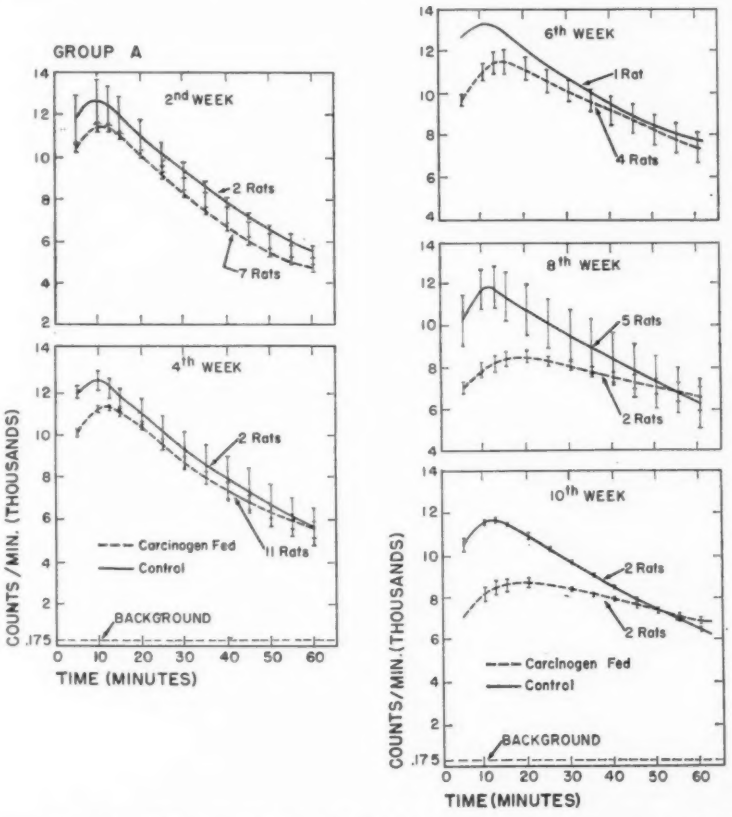


TEXT-FIGURE 1.—Tracings of radioactivity over liver area, obtained from a single carcinogen-fed rat in group A at the 2nd (*left*) and 8th (*right*) week of the experiment. On a scale of 15,000, each 50 units on the horizontal lines is equivalent to 1,500 counts. Vertical lines represent time intervals of 15 minutes, with recording chart moving from *right* to *left*. All curves constructed from such tracings have been reversed for the reader's convenience.

constructed to read from left to right for convenience of interpretation. The vertical bars indicate standard errors calculated by the method of Mantel (*10*).

The mean uptake-excretion curves of the carcinogen-fed rats of both groups A and B (text-figs. 2 and 3) show in general a progressive decrease in rate of excretion of radioactive material from the liver. The tendency toward slower excretion begins for both groups in the fourth week. It becomes markedly "abnormal" for group B in 6 weeks and for group A in 8 weeks. The difference in counts per minute between 10 and 60 minutes dropped from 6,700 in the second week to 1,300 in the eighth week for group A, and from 5,500 in the second week to −700 in the sixth week for

group B (text-fig. 4). To express these changes in excretion of radioactive material more succinctly these curves were replotted on semilog paper (not illustrated). The descending part of such semilog curves is a straight line whose slope is the percent rate of disappearance of the radioactive dye from the liver. These constants, when plotted against time in weeks



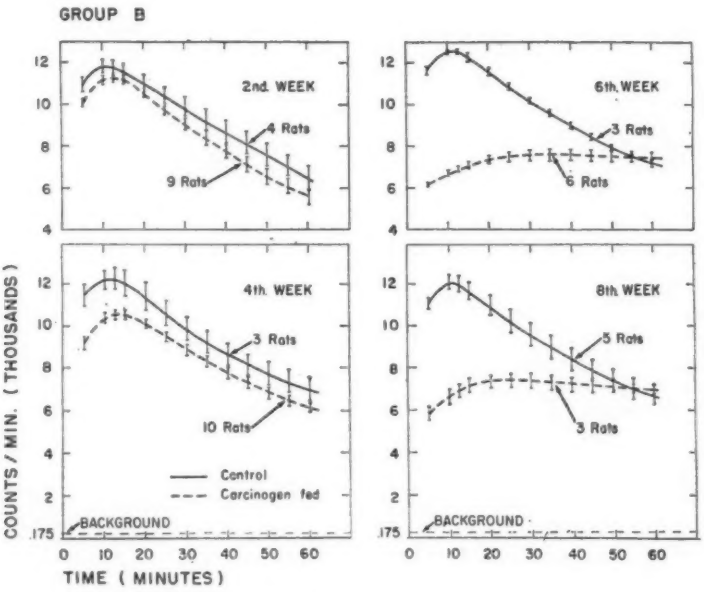
TEXT-FIGURE 2.—Mean 60-minute radioactivity uptake-excretion curves of carcinogen-fed and control rats in group A for each biweekly test period. Vertical lines indicate standard errors. The part of the curves between 0 and 5 minutes was not plotted because uptake was so rapid at this time.

(text-fig. 5), provide a graphic summary of the changes in rate of excretion of radioactive material during the 8 or 10 weeks of the experiment.

The average height of the peak of radioactivity (text-fig. 4) progressively decreased for carcinogen-fed rats of group B—slightly in the fourth week and significantly during the sixth week. The mean values were 11,500, 10,700, and 7,600 counts per minute for the second, fourth, and sixth weeks, respectively. The peak values for group A were noticeably lower at the eighth and tenth weeks but not for the earlier periods studied.

Thus the peak activity dropped from 11,500 in the sixth week to 8,500 and 8,700 counts per minute in the eighth and tenth weeks, respectively.

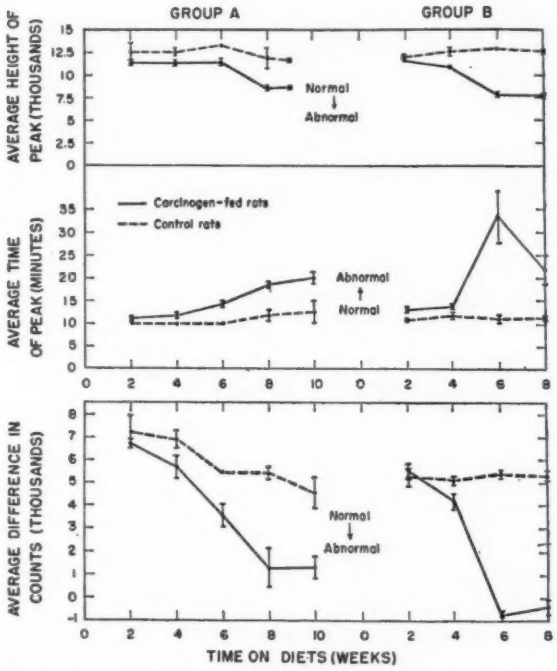
Though the uptake of dye (as measured by radioactivity) in all groups at all times was initially very rapid, a delay in the time at which the peak was reached began in the sixth week for both groups. The average values range from 11.4 minutes in the second week to 20.0 minutes in the tenth week for group A, and from 12.8 minutes in the second week to 33.3 minutes in the sixth week for group B (text-fig. 4). If the maximum count (55 minutes) for one of the rats from the 6-week group is eliminated, the average time for this group to reach the peak is reduced to 29 ± 3



TEXT-FIGURE 3.—Mean 60-minute radioactivity uptake-excretion curves of carcinogen-fed and control rats in group B for each biweekly test period. Vertical lines indicate standard errors. The part of the curves between 0 and 5 minutes was not plotted because uptake was so rapid at this time.

minutes. Since the first or rapid-uptake phase remained unchanged in carcinogen-treated animals, the delay in reaching a maximum count was due to a decreased rate in the second portion of the uptake period (text-fig. 1).

The average rates of uptake, maximum uptakes, and excretion rates of radioactivity for the control animals of group A tended to parallel those of the group A carcinogen-fed rats. The control values, however, showed much smaller changes at the successive biweekly test periods than those of the treated animals. The controls of group B maintained uniform values throughout the experiment.



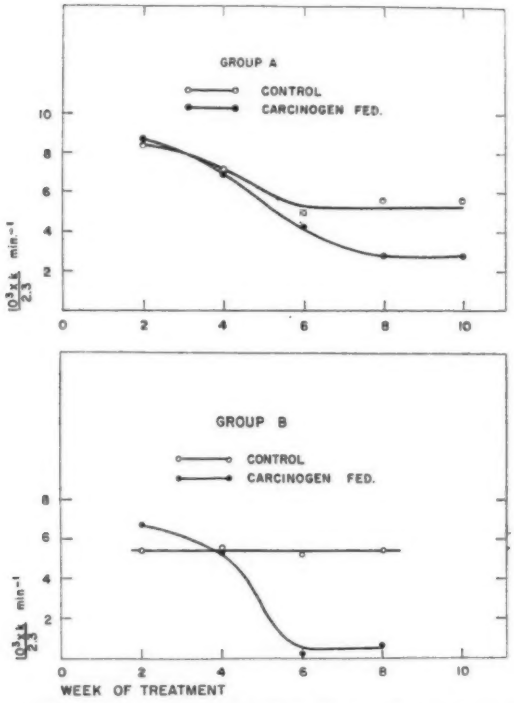
TEXT-FIGURE 4.—Comparison of response of control and carcinogen-fed rats in group A (*left*) and group B (*right*) to the rose-bengal I^{131} liver-function test at biweekly intervals. Vertical lines indicate standard errors. *Upper section*: average height of peak in counts per minute, indicating mean total uptake of radioactivity by the liver. *Middle section:* average time at which peak was reached, indicating uptake time. *Lower section:* average difference between 10- and 60-minute counts, indicating amount of excretion.

Pathological Gross Examination

Group B Rats

1) *On experimental diet #284 containing* N-*2-fluorenyldiacetamide.*—No significant changes were observed on gross examination in the group B rats that were killed after 2 weeks on diet #284. Pallor noted in the livers after 4 weeks was attributed to extensive fatty change. The livers of rats killed after 6 weeks contained several small nodules that were raised above the surface (*9*) and randomly distributed among the hepatic lobes. The largest nodules measured 3.0 mm. in diameter. The livers of the rats killed at 8 weeks were coarsely nodular, the individual nodules being larger than those seen at 6 weeks, with the largest nodule measuring 5 mm. in diameter.

2) *On control diet #285.*—Some of the livers of the rats fed the control diet were pale and some contained hemorrhagic areas. The changes that were noted did not progress with time on the diet but occurred in



TEXT-FIGURE 5.—Slopes of the semilog plots of text-figure 4 as functions of the number of weeks of treatment. As indicated by the ordinate, k, the percent rate of change of the counts per minute in a given test can be calculated by dividing a slope by 23,000.

sporadic cases among the animals killed at the biweekly intervals over an 8-week period.

Group A Rats

1) *On experimental diet #284.*—The group A rats ingesting the carcinogen for 10 weeks and receiving 4 and 5 test doses of radioactive rose bengal at biweekly intervals had liver lesions similar to those described for the rats in group B at 8 weeks, except that the nodules were larger and more numerous. Some of the nodules measured 9 mm. in diameter. Most of the hepatic tissue was replaced or obscured by the nodules.

2) *On control diet #285.*—The group A rats on the control diet for 10 weeks and also receiving 4 test doses of radioactive rose bengal at biweekly intervals had liver lesions similar to those seen in the control rats of group B studied from 2 to 8 weeks. The gross changes were not more severe in this group than in those of group B on the same diet.

Histopathology

The most significant changes in the livers of rats used in this experiment appeared to be: 1) alterations in the fat within the hepatic cells,

2) clumping of basophilic cytoplasmic material or cytoplasmic basophilia, 3) appearance of hyaline bodies, 4) development of pseudotubules, 5) appearance of lesions around the portal triads, 6) development of hyperplastic hepatic nodules, and 7) carcinomas.

These lesions are briefly described as follows:

1) *Changes in fat content of the hepatic cells.*—This was usually demonstrated as fat droplets of varying sizes by the oil red O stain. In hematoxylin-and-eosin-stained sections the change was represented by vacuolation.

2) *Basophilic material within the liver-cell cytoplasm.*—This varied from faintly discernible basophilic granules to large aggregations in masses or linear arrangements. The basophilic material stained blue with hematoxylin-eosin-azure, deep pink with hematoxylin and eosin, purple with Masson's trichrome, and blue-black with Regaud's hematoxylin. The basophilic material was sparse in vacuolated hepatic cells. When the basophilic material appeared in large masses or in linear arrangement, it was usually found near the cell border.

3) *Hyaline bodies within the cytoplasm of the hepatic cells.*—Pale-staining, oval or crescent-shaped acidophilic hyaline structures were observed in the cytoplasm of hepatic cells. They usually occupied a space between the nucleus and the cytoplasmic basophilic material and sometimes partially or entirely encircled the nucleus. These bodies stained light green with Masson's trichrome stain and pale blue with hematoxylin-eosin-azure.

4) *Pseudotubules.*—Few to many tubular or ductlike structures appeared in the periportal areas and in the liver lobules among the hepatic cords, which they appeared to replace in some areas. They appeared as a double row of fusiform to cuboidal pale-staining cells that occasionally were in direct continuity with lumenized tubular structures composed of similar cells. These structures have been called pseudotubules (*11*). The cells of the pseudotubules contained no cytoplasmic basophilic granules as demonstrated by phosphotungstic acid-hematoxylin, and no glycogen as demonstrated by periodic-acid leucofuchsin technique after diastase digestion. These pseudotubules lay in a fine network of fibrils. In the periportal area they were arranged radially about the portal vein and were usually more numerous on the side of the portal vein opposite the satellite bile duct.

5) *Portal triad lesion.*—This lesion in the periportal spaces consisted of thickening of the walls of the vein and artery, dilation of lymph vessels, fibrosis, and inflammatory cell infiltration.

6) *Hyperplastic hepatic nodules.*—The nodules were well-circumscribed spherical areas of hepatic-like cells that were differentiated from the surrounding hepatic tissue by the strong basophilia of the cells and by the enlargement of the hepatic cells. The glycogen content of the cells within the nodules was evenly distributed throughout and the amount was greater than that in the surrounding hepatic cells. An increased amount of connective tissue immediately surrounding the spherical areas was not apparent until the sixth week of carcinogen feeding. Mitotic figures were found more frequently in the nodules than in the surrounding hepatic

tissue. Variations in the size and staining characteristics of the nuclei were few in some and many in others. The factors taken into consideration in making the differentiation between hyperplastic and neoplastic nodules are considered under the heading of carcinomas.

7) *Carcinomas.*—Circumscribed spherical areas considered to be carcinomas were differentiated from hyperplastic hepatic nodules by the disorderly arrangement of the cells, the presence of cords several cells thick, and by the absence of portal triad structures and of Kupffer cells. The neoplastic cells showed marked variability in size and staining characteristics. There was less fatty change and greater variability in the glycogen content in the carcinomas than in the hyperplastic nodules.

The neoplasms of the liver induced by the oral administration of carcinogenic agents have been thoroughly described in the literature (*12*) and it is generally agreed that it is difficult to determine whether some lesions are neoplastic or hyperplastic. In instances in which the variations in size and staining characteristics of the nuclei were great, and where considerably thickened cords of hepatic cells were present, it was difficult to determine whether such nodules represented hyperplastic or neoplastic lesions. In questionable cases the nodules were considered hyperplastic.

Other infrequent lesions appeared only in rats on the diet for the longer periods of time and will be described along with other changes in the livers of these rats examined at biweekly intervals.

Group B Rats on Experimental Diet #284 at the Intervals Examined

1) *Two weeks.*—A slight to moderate fatty change was seen in the central lobular hepatic cells (fig. 1). In the peripheral and midzones the basophilic cytoplasmic granules appeared in large masses (fig. 2) and less often in linear aggregates lying close to the cell membrane (fig. 3). The granules were finer and less basophilic in the central zones. Few to many hyaline bodies occurred in the mid- and peripheral zones of most liver lobules (fig. 4). Several pseudotubules appeared in the periportal areas (figs. 5 and 6) and at the periphery of many liver lobules. The walls of the vessels in the portal triad were thickened, particularly the portal vein (fig. 7). Lymph vessels were occasionally distended and contained a pale-staining homogeneous material. Inflammatory cell foci appeared occasionally in the periportal areas.

2) *Four weeks.*—The fatty change was more extensive in the central portions of the liver lobules than at 2 weeks. The change occasionally involved the midzones of the lobules. The clumping of cytoplasmic basophilic granules was also somewhat more marked in the mid- and peripheral zones of the lobules than that seen at 2 weeks. Pseudotubules were numerous whereas at 2 weeks there were only a few. They appeared in the periportal areas, frequently circumscribed liver lobules, and were seen lying between the hepatic cords in the peripheral zones of the lobules. The periportal spaces were more prominent at 4 than at 2 weeks. The portal triad area showed an increase in reticular and collagenous connective tissue, moderate thickening of the vessel walls, and dilatation of lymph vessels.

The number of inflammatory cell foci were approximately the same at 2 and 4 weeks.

Nodules of altered liver cells were first observed at 4 weeks (fig. 8). These were poorly circumscribed spherical areas of hepatic-like cells with strongly basophilic cytoplasm arranged in cords of 2 or more cells thick. A few of these were seen scattered throughout the liver sections of 4 of the 10 animals (table 1).

3) *Six weeks.*—The fatty change varied from moderate to extensive in the central lobular areas, but was less marked in cells in the center of the lobule than the change seen at 4 weeks. Atrophy and degeneration were occasionally observed. Clumping of cytoplasmic basophilic material was most pronounced in the cells of the midzones, but fewer cells were involved than at any other interval. The basophilic granules stained faintly in the hepatic cells in the peripheral zones of the lobules. Pseudotubules were more abundant in the peripheral portions of the lobules at 6 than at 4 weeks and they frequently replaced almost all hepatic cells in the peripheral one third of the lobules (fig. 9). Reticular and collagenous fibers accompanied the pseudotubules and were present in the mid-portions of some of the lobules, resting between the hepatic cells and the pseudotubules.

Several well-circumscribed spherical areas of hepatic-like cells were observed (figs. 10 and 11). They were more numerous at 6 than at 4 weeks. Some of these nodules contained cords several cells thick and the cells showed marked variability in size and staining characteristics. These were considered to be carcinomas. Other spherical areas similar to those described at 4 weeks were seen. Some of these could not be distinguished from hyperplastic hepatic nodules.

A few multilocular cystic areas were noted for the first time (fig. 12). The cysts were lined with cells having very scant eosinophilic cytoplasm and containing oval, vesicular nuclei. The periportal spaces contained numerous reticular and collagenous fibers. The walls of the vessels of the portal triads were thickened. Lymph vessels were distended. A pale-staining, hyaline-like material appeared outside the wall of the portal veins and lymph vessels. The lesions in the portal triad area did not appear to be more marked at 6 than at 4 weeks.

4) *Eight weeks.*—The fatty change was much less at 8 than at 6 weeks and appeared only in scattered hepatic cells. The basophilic cytoplasmic granules were poorly stained in the majority of cells. Clumping of the basophilic material occurred, but to a lesser degree than at 6 weeks. Cytoplasmic hyaline bodies were so few and ill-defined, in comparison with those seen at 6 weeks, that they were not noted in table 1.

Pseudotubules, which were almost as numerous at 8 as they were at 6 weeks, separated the residual hepatic cells into small groups. They were almost always markedly compressed by carcinomas and hyperplastic nodules (fig. 13), which were more numerous and larger than those seen at 6 weeks and replaced the greater part of the liver tissue. The pseudotubules were associated with a considerable amount of reticular and

TABLE 1.—*Radioactivity uptake-excretion readings and histopathologic changes of the livers of rats fed N-2-fluorenyldiacetamide (diet #284) and control diet (#285) and sacrificed at intervals of 2 weeks*

		Readings of radioactivity			Histologic findings											
	Animal No.	Weeks on diet	Δ counts 10 to 60 minutes (*thousands*)	Height of peak (*thousands*)	Time of peak (*minutes*)	Clumped cytoplasmic basophilic nuclei*	Hyaline bodies*	Fatty change*	Pseudotubules*	Fibrosis*	Hepatic cell degeneration†	Hepatic cell necrosis†	Hyperplastic nodules†	Carcinomas†	Multilocular cysts†	Adenofibrosis†	Portal triad lesions†	Inflammatory cell foci*	Residual hepatic tissue (%)
Animals on diet #284 Group B	1	2	6.6	11.1	10	+	+	+	+									+	100
	2		5.8	10.5	15	+	+	+	+									+	100
	3		6.2	11.3	10	+	+	+	+									+	100
	4		5.8	11.7	10	+	+	+	+									+	100
	5		6.4	12.0	10	+	+	+	+									+	100
	6		5.0	11.7	15	+	+	+	+									+	100
	7		3.8	11.3	20	+	+	+	+									+	95
	8		6.2	11.3	10	+	+	+	+		+	+					+	+	100
	9		4.0	12.3	15	+	+	+	+									+	100
	Avg.	4	5.5	11.5	12.8	+	+	+	+	+	+	+	+					+	70
	10		4.1	9.7	12.5	+	+	+	+	+	+	+						+	50
	11		2.0	9.7	20	+	+	+	+	+	+	+	+					+	60
	12		3.1	10.1	15	+	+	+	+	+	+	+						+	60
	13		2.6	10.8	15	+	+	+	+	+	+	+						+	60
	14		5.4	11.2	10	+	+	+	+	+	+	+						+	80
	15		5.5	10.8	10	+	+	+	+	+	+	+						+	80
	16		4.6	10.6	15	+	+	+	+	+	+	+						+	50
	17		4.9	11.3	10	+	+	+	+	+	+	+						+	80
	18		4.9	11.5	15	+	+	+	+	+	+	+	+					+	60
	19		5.3	11.5	12.5	+	+	+	+	+	+	+						+	60
	Avg.		4.2	10.7	13.5	+	+	+	+	+	+	+						+	80
	20	6	−0.4	7.0	20	+	+	+	+	+	+	+	+	+	+		+	+	40
	21		−0.9	7.3	55				+	+	+	+	+				+	+	30
	22		−0.6	7.6	30				+	+	+	+	+				+	+	40
	23		−0.6	7.6	30				+	+	+	+	+	+			+	+	40
	24		−1.2	8.5	35				+	+	+	+	+	+			+	+	25

25		−0.5	7.7	30	+	+	++	+++	++	+	+	+++	+	+		++	+	40
Avg.		−0.7	7.6	33.3														
26	8	0.2	7.6	15	+		+	+++	+++			++++	+	++	+	++	+	15
27		−0.8	7.0	25	+		+	+++	+++			+++	++	++		++	+	10
28		−0.5	8.1	25	+		+	+++	++			+++	++	++		++	+	20
Avg.		−0.4	7.6	21.7														
Group A																		
29	10	1.8	8.8	15	+			++	+++			++++	++++	+++	+	++	++	10
30		0.8	8.6	25				++	+++			++++	+++	+++		++	+	15
Avg.		1.3	8.7	20														
Animals on diet #285																		
Group B																		
31	2	6.0	11.4	10			++++			+	+					+	+	
32		4.5	12.1	10			+++			+	+					+	+	
33		5.2	12.6	10			+			+	+					+	++	
34		5.5	11.4	12.5			+++			+	+					+	+	
Avg.		5.3	11.9	10.6														
35	4	4.9	12.0	12.5			++			+	+					++	+	
36		5.5	11.8	10			++									++	+	
37		5.0	13.2	12.5			++			+	+					+	+	
Avg.		5.1	12.3	11.7														
38	6	5.7	12.7	10			+									+	++	
39		5.2	12.7	12.5			++									++	+	
40		5.4	12.6	10			+++									++	+	
Avg.		5.4	12.7	10.8														
41	8	6.0	11.8	10			++									++	+	
42		4.9	13.0	12.5			+									+++	+	
43		5.1	12.5	12.5			++++			+	+					+	+	
44		5.1	11.4	10			++++									++	+	
45		5.8	12.4	10			+									+	+	
Avg.		5.4	12.2	11.0														
Group A																		
46	10	3.9	11.5	12.5			+			+	++					++	+	
47		5.2	11.7	12.5			++									++	+	
Avg.		4.5	11.6	12.5														

Extent of lesions: + few scattered cells involved (slight); ++ moderate, involving ¼ to ½ of a liver lobule; +++ marked, involving ½ to ¾ of a liver lobule; ++++ extensive, involving ¾ to all of a liver lobule of the majority of lobules in sections.

† Number of such lesions per 100 low-power fields: + 1 to 20; ++ 20 to 40; +++ 40 to 60; ++++ 60 to 80.

collagenous connective tissue, a few inflammatory cells, and some histiocytes containing a canary-yellow pigment considered to be ceroid (*13*) (fig. 14). This pigment was periodic-acid leucofuchsin positive after treatment of the sections with diastase and acid-fast with Verhoeff's method.

Multilocular cysts were more numerous at 8 than at 6 weeks. For the first time an occasional small area of adenofibrosis (cholangiofibrosis) was observed (table 1). The lesion consisted of glandlike structures surrounded by dense collagenous and reticular connective tissue (fig. 15). The lumens of the glandlike structures were lined with epithelial cells containing a mucoid material that, after diastase digestion, was positive with the periodic-acid leucofuchsin technique.

Group B Rats on Control Diet #285

1) *Two weeks.*—A slight to extensive fatty change was noted which was usually limited to the peripheral lobular hepatic cells (fig. 16). A few scattered hepatic cells at the periphery of lobules showed degeneration and necrosis.

2) *Four weeks.*—The fatty change involved approximately the peripheral one fourth to one third of the majority of lobules. The lesions in the periportal spaces or portal triads were somewhat more pronounced at 4 than at 2 weeks.

3) *Six weeks.*—The fatty change varied from slight to moderate; it usually involved the same peripheral portion of the lobule to the same extent as at previous intervals. The lesions in the periportal spaces were similar in nature and extent to those observed at 2 and 4 weeks.

4) *Eight weeks.*—The location and extent of the fatty change was like that described at earlier intervals. Occasional necrotic foci and areas of hemorrhage were seen. The lesions of the periportal spaces were like those described at 6 weeks, but they appeared to be slightly more numerous in a few animals.

Group A Rats on Experimental Diet #284

Ten weeks.—There were few residual hepatic cells in the livers, and these were compressed between the neoplastic and hyperplastic nodules (fig. 17). A few of them were vacuolated, while others showed degeneration and necrosis. A brown, iron-containing pigment was seen in some liver cells. Relatively less normal hepatic tissue was seen at 10 weeks in the group A rats than at 8 weeks in the group B rats. The residual hepatic cells contained fine cytoplasmic basophilic granules that occasionally were clumped and that stained faintly with basophilic dyes.

Numerous pseudotubules circumscribed the hepatic nodules and were compressed by them. The pseudotubules appeared to be fewer at 10 weeks in the rats of group A than at 8 weeks in those of group B. The relative paucity of pseudotubules may have been due to the compression of these structures by hepatic nodules. The amount of collagenous and reticular connective tissue seen in the livers of rats in this group was greater than that seen in the livers of the rats of group B at 8 weeks.

The hepatic nodules were larger and more numerous than those seen in group B at 8 weeks. More carcinomas were also present than were seen at previous intervals in the group B rats, and the carcinomas frequently contained glandular elements (figs. 18 and 19). Small multilocular cysts were seen like those described previously but were more numerous. A few areas of cholangiofibrosis, slightly more numerous than in group B rats, were seen. The lesions of the portal triads were similar in character and extent to those described for group B rats at 8 weeks.

Group A Rats on Control Diet #285

Ten weeks.—The fatty change was slight to moderate; it was usually limited to the peripheral parts of the lobules, though a few areas of centrolobular vacuolation were noted. Several foci of necrosis and a few areas of degeneration were scattered throughout the lobules.

The lesions of the portal triads were similar in character and extent to those described in the livers of control rats in group B at 8 weeks.

Summary of Microscopic Findings

The fatty change in the hepatic tissue occurred in animals on both diets. The change was usually in the central portions of the lobules in the rats on the carcinogen-containing diet #284, whereas it was in the peripheral parts of the lobules in animals on the control diet #285. The fatty change in the livers of the animals receiving the carcinogen was more extensive at 4 than at 2 weeks, but decreased thereafter in proportion to the decrease in the number of residual hepatic cells. No such relationship to time was observed in the control animals, though the fatty change varied from slight to extensive.

The degree of clumping of the cytoplasmic basophilic material in hepatic cells progressively decreased from the second to the eighth week and paralleled a decrease in the depth of the basophilic-staining capacity of the hepatic cells. The cytoplasmic basophilic material and the number of cytoplasmic hyaline bodies diminished correspondingly as the number of residual hepatic cells decreased. The pseudotubules, which appeared first at 2 weeks and increased in number to reach a peak at 6 weeks, were associated with a corresponding increase in collagen and reticulum, though the amount of the latter was less noticeable. The pseudotubules were approximately the same in number at 6 and 8 weeks. Hyperplastic nodules were first noted at 4 weeks and increased in size and number up to the eighth week. The number of nodules composed of carcinoma cells also increased, with time on the diet, from the sixth to the eighth week. Necrosis and degeneration of hepatic cells were noted particularly at the fourth and sixth week. Multilocular cystic areas were observed at the sixth and eighth week.

The amount of residual hepatic tissue decreased progressively from the second to the sixth week on the carcinogenic diet and was gradually replaced by pseudotubules, connective tissue, hyperplastic hepatic nodules, and carcinomas.

Similar changes were not observed in the livers of rats on the control diet #285. Although the fatty change did occur, it took place in the peripheral rather than the central part of the lobule. The repeated administration of radioactive rose bengal did not appear important etiologically in the formation of the portal triad lesions because such lesions were not more extensive for group A animals on either diet than they were for group B animals. The basic diet, however, may have influenced the development of the portal triad lesions because they appeared to become progressively more noticeable with time on the diet.

The livers of group A rats on the experimental diet #284 for 10 weeks showed lesions like those described for group B animals on the same diet for 8 weeks, except that the number and the size of the carcinomas and hyperplastic nodules were greater and the amount of residual hepatic tissue was less in the rats on the diet for 10 weeks.

Comparison of Stages of Liver Lesions With Functional Changes

1) *Fatty change.*—This change did not appear to affect the rose-bengal test unless it was associated with degeneration and necrosis of hepatic cells. A drop in the Δ count, a lower peak, and a delayed peak time were seen in association with fatty change, degeneration, and necrosis of hepatic cells for animal 7 at the second week, and in animals 10, 11, 12, 13, and 16 in the fourth week (table 1). All animals in the sixth week had similar changes; the fatty change, however, involved fewer hepatic cells at this time. It was questionable whether the necrosis and degeneration were sufficiently great to be significant factors in altering the test readings, even though there appeared to be some correlation. Lesions other than fatty change, degeneration, and necrosis of hepatic cells were considered more important factors in altering the test readings.

2) *Cytoplasmic basophilic changes.*—The gradual decrease in the degree of clumping and in the amount of cytoplasmic basophilic material paralleled the degree of alteration of the test readings, but this was believed to be related to the progressively decreasing number of residual hepatic cells with time on the carcinogenic diet.

3) *Hyaline bodies.*—Hyaline bodies were most numerous in instances where the degree of clumping of the cytoplasmic basophilic material was greatest, and the relation to the function test is like that described for the cytoplasmic basophilic material.

4) *Pseudotubules.*—The increasing number of pseudotubules roughly paralleled the decreasing absorption and excretion of the radioactive dye by the liver. During the 6- and 8-week intervals, when the radioactive rose-bengal tests indicated the greatest functional impairment, the pseudotubules were most numerous, and as they increased in number they replaced the peripheral parts of most liver lobules. An increase in collagen and reticulum was also associated with the increase in pseudotubules.

5) *Portal triad lesions.*—The portal triad lesions increased progressively in group B rats from the fourth to the eighth week, and at 8 weeks were, in extent, like those seen in group A at 10 weeks (table 1). Animals

42 and 44 of the 8-week group had lower Δ counts and more extensive portal triad lesions than animals 41 and 45, but animal 43 had the same Δ counts as 44 with fewer triad lesions. It would seem that the correlation between the rose-bengal test and portal triad lesions, while positive, was only partially so.

6) and 7) *Hyperplastic hepatic nodules and carcinomas.*—One of the main purposes of the rose-bengal test in the present studies was to determine whether a close correlation existed between the functional test and the development of neoplastic nodules. As the hyperplastic and neoplastic nodules increased in size and number and replaced the residual hepatic tissue, the radioactive rose-bengal test readings became progressively lower (table 1). Animals that showed poorly circumscribed, spherical areas of altered hepatic cells at 4 weeks were those with the most abnormal readings. Their Δ counts were very low and the time to reach the peaks was delayed (table 1). The liver-function tests of group B rats at 8 weeks might be interpreted as indicating slightly better function, yet the morphologic findings indicated more and larger hyperplastic nodules and carcinomas than at 6 weeks. One possible explanation could be that the hyperplastic nodules were capable of absorbing some of the dye.

In the 2 carcinogen-fed group A animals that were tested for 4 and 5 biweekly intervals and killed after the last test, the average Δ readings (text-fig. 2) indicated progressively decreasing ability of the liver to absorb the dye through the tenth week. The microscopic changes were like those in the livers of the rats of group B, except that the hyperplastic hepatic nodules and carcinomas were more numerous and larger with less residual hepatic tissue in the livers.

Comparison Summary

As the residual hepatic tissue was compressed and separated from its normal relationship to vascular structures, and as the parenchyma was replaced by pseudotubules, connective tissue, hyperplastic hepatic nodules, and carcinomas, the functional test readings became progressively more abnormal. The effect of other changes, such as fatty alteration, degeneration and necrosis, clumping and reduction in the amount of cytoplasmic basophilic material, and appearance of hyaline bodies, could not be adequately evaluated on the basis of the present data.

Discussion

These experiments, in general, have demonstrated a good correlation between the results of the rose-bengal I^{131} liver-function test and the development of liver neoplasms in rats ingesting *N*-2-fluorenyldiacetamide. The test indicated an impairment in liver function before carcinomas could be recognized. By the time tumors had developed in the livers of group B rats, the test always showed impairment of ability to take up and excrete the radioactivity of the dye. The total uptake of group B rats at the sixth week seldom exceeded 60 percent of that of the

controls, and none of the tests showed a liver clearance of over 10 percent of the peak number of counts; some of the tests showed virtually no excretion during the period of the test.

A decision as to the importance of some of the morphologic changes occurring during the ingestion of *N*-2-fluorenyldiacetamide in the process of hepatocarcinogenesis was difficult because morphologic changes also occurred in the control group. When the diet contained *N*-2-fluorenyldiacetamide (*9*), it was known to induce liver tumors rapidly and abundantly. Fatty change, degeneration and necrosis of hepatic cells, and portal triad lesions also appeared in the livers of animals on the control diet. These lesions therefore were probably of dietary origin, though they could not be attributed to any one dietary factor. However, they did not appear to alter the function test. The diet was low in protein and fat, and no choline was added because it was thought that 12 percent casein provided adequate methionine to permit the formation of minimal amounts of choline. However, the numerous histologic changes in the livers of the rats on the diet containing *N*-2-fluorenyldiacetamide made it impossible to select a single change as the only factor determining the results of the function test. The histologic changes which were most closely associated with ingestion of the carcinogen and which appeared to influence the function-test readings most directly were the pseudotubules and the hepatic nodules, either hyperplastic or neoplastic. The smaller the amount of residual hepatic tissue, the more abnormal the radioactivity readings, and this paralleled the replacement of hepatic cells by pseudotubules and hepatic nodules.

Actually, combinations of several abnormalities plus neoplastic changes were probably responsible for the striking loss of function as revealed by the dye test. Some of these abnormalities may explain the slight impairment at the fourth week before there was clear morphologic evidence of neoplasia. The possibility must also be considered that the ingested *N*-2-fluorenyldiacetamide altered the metabolism of many liver cells and decreased slightly the amount of dye that each cell could take up and eliminate before morphologic changes were evident.

Additional studies are needed to determine the possible influence on liver function of other alterations, such as changes in the amount and distribution of cytoplasmic basophilic material and of hyaline bodies. The independent effects of the pseudotubules, hyperplastic nodules, and carcinomas could only be studied if they could be induced separately. This might be possible, in some degree, in a more slowly developing process of tumor formation.

Although the carcinogen-fed rats of groups A and B both showed the same qualitative changes in radioactive rose-bengal uptake-excretion pattern, we cannot explain the difference in the time of appearance of the changes between the 2 groups nor the differences in the absolute values of the variables measured (text-figs. 2 and 3). Also, it is not at all clear why the controls of the 2 groups were not alike in their response to the test at 2 and 4 weeks. Every effort was made to treat all animals in

exactly the same way. The one obvious difference in treatment was that all group A rats received several injections of radioactive rose bengal, one at each test period; each rat in group B received one injection and was then sacrificed. Some of the differences between the 2 groups in the results of the functional test were probably due to the fact that only a small number of animals survived to receive the later tests.

The meaning of the pattern of response to the test, as revealed in the shape of the tracings (text-fig. 1), is still a matter of conjecture. Although these experiments were not designed to study the physiological aspects, the changes in rate of uptake and excretion reflected in the tracings may be significant. One possible explanation is that during the very rapid initial uptake in both normal and abnormal livers the radioactive dye in the blood vessels of the liver was being measured. A decrease in uptake rate would occur as the dye began to enter the cells, and if fewer cells were capable of accepting the dye, the decrease in the rate of uptake would be even more marked. Rose bengal is believed to be bound by serum proteins (*14*). If intracellular protein binding of some of the dye molecules also occurred, such bound dye molecules or serum-bound dye molecules in the damaged liver would be eliminated more slowly. Thus, if the unbound dye had been excreted rapidly, the rate could, at least partially, depend on the release of the bound dye and this might be reflected in the later slow phase of excretion.

Many investigators have studied early morphologic changes in livers of rats during hepatocarcinogenesis, but no previous attempt has been made in experimental animals to correlate a function test with such structural changes. Price *et al.* (*15*) described cytoplasmic hyaline bodies in hepatic cells after only 7 days of azo-dye ingestion. They postulated that the lesion represented a morphologic manifestation of the protein-bound azo dye in the cytoplasm of the hepatic cells. This was substantiated by a close correlation between the lesions and the amount of the dye levels in the livers. They found that the liver parenchymal cells vanished as the proliferating bile ducts increased. Bile-duct proliferation was seen by them first at 18 to 24 days, and it had extended nearly to the central veins of most lobules by the 26th to 28th day. In the animals that died, or were killed when moribund, bile-duct proliferation was extensive and no hyaline bodies were found. Price *et al.* concluded that death was due to hepatic insufficiency because the bile-duct cells and the parenchymal cells which they surrounded were incapable of supporting vital hepatic functions.

Spain (*16*) reported that many of the changes observed in biochemical studies on livers of rats that had been treated with azo dyes for at least 3 weeks were the result of "diluting" parenchymal tissue with that of the bile-duct cells.

Furthermore, the observations of Price *et al.* (*15*), that the livers of animals dying with hepatic insufficiency contain abundant bile-duct structures, suggest that similar structures observed in the livers of rats fed *N*-2-fluorenyldiacetamide may have been an important influence in alter-

ing the function test. The abnormal counts obtained at 4 and 6 weeks occurred at times when the pseudotubules were most abundant. The functional alteration could be attributed in part to the replacement of hepatic cells by pseudotubules and in part to the obstruction of the proper functioning of the residual hepatic cells by cords of pseudotubules and fibrous-connective tissue which separated hepatic cells into small groups.

Hartroft and Ridout (*17*) considered that there was a relationship between the appearance of increased numbers of bile ducts and multilocular fatty cysts in the livers of rats fed a diet low in choline. They reported that multilocular fatty cysts always preceded the appearance of increased numbers of bile ducts, and that conglobation of fat droplets occurred in livers showing centrolobular fatty changes. In the livers of rats that received *N*-2-fluorenyldiacetamide in the present experiment, the pseudotubules appeared approximately 4 weeks before the multilocular cysts were seen. Furthermore, similar structures were not seen in the livers of rats on the control diet; therefore, their appearance could not be attributed directly to the diet.

Spain and Griffin (*18*) observed a depletion of liver glycogen in the centrolobular zone in the livers of rats that had been fed *N*,*N*-dimethyl-*p*-(*m*-tolylazo) aniline (3′-methyl-4-dimethylaminoazobenzene) for at least 14 days. They interpreted the lessened ability of the cells in the central zone to carry out the synthesis of glycogen as a central zone anoxia caused by toxic effects of the azo dye. Similar changes were observed by Spain and Griffin to result from the administration of ethionine, carbon tetrachloride, and, to a lesser degree, from 4-dimethylaminoazobenzene but not from several noncarcinogenic dyes that were tested. In the livers of rats fed *N*-2-fluorenyldiacetamide in these experiments, no depletion of glycogen in the central zone was seen; pseudotubule formation, however, had already begun at the second week. If this change did occur with the carcinogen used here, it was missed because tissues were not examined early enough, *i.e.*, prior to 14 days.

In our opinion, the radioactive rose-bengal test shows considerable promise as a means of detecting early changes in liver function during the ingestion of a carcinogen under the conditions of these experiments, making it unnecessary to kill the animals or to perform biopsies. The fact that so little dye is necessary to detect changes in uptake and excretion attests to the sensitivity of the radioactive rose-bengal test and increases its value.

Summary

A radioactive rose-bengal liver-function test was performed on A × C 9935 strain male rats receiving the carcinogen *N*-2-fluorenyldiacetamide. A collimated scintillation counter was positioned directly over the liver area, the dye injected intravenously, and the I^{131} activity continuously recorded during a 60-minute period. The test was repeated on the same rats in one group after 2, 4, 6, 8, and 10 weeks. Animals of another group were tested at 2, 4, 6, and 8 weeks—each rat being sacrificed immediately after its test.

The results of the functional tests on carcinogen-fed rats, in comparison with controls, showed progressive changes with time as follows: 1) radioactivity clearance rate decreased, beginning in the fourth week, as the first indication of impaired ability to eliminate the dye from the liver; 2) a delay in the uptake of radioactivity from the blood began to occur at the sixth week; and 3) the total radioactivity uptake by the liver had diminished significantly at 6 or 8 weeks, with little or no liver clearance taking place.

Microscopic changes occurring in liver sections at the several intervals studied during hepatocarcinogenesis in rats fed *N*-2-fluorenyldiacetamide have been compared with counts of radioactivity indicative of absorption and excretion of the radioactive rose-bengal dye. The influence on the function test of fatty change, degeneration and necrosis of hepatic cells, alterations in the amount and distribution of cytoplasmic basophilic material, and the occurrence of cytoplasmic hyaline bodies was studied and discussed but could not be fully evaluated. As the hepatic tissue was replaced by pseudotubules and connective tissue, hyperplastic nodules, and carcinomas, the radioactivity measured over the liver indicated less absorption and retarded excretion of the dye. Striking impairment of liver function occurred at the time of the appearance of carcinomas. Control livers manifested no neoplastic changes.

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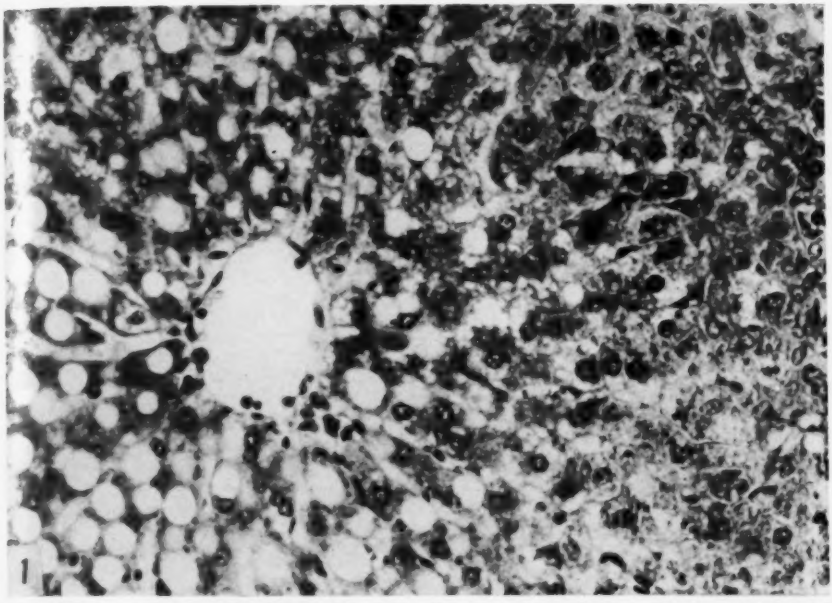
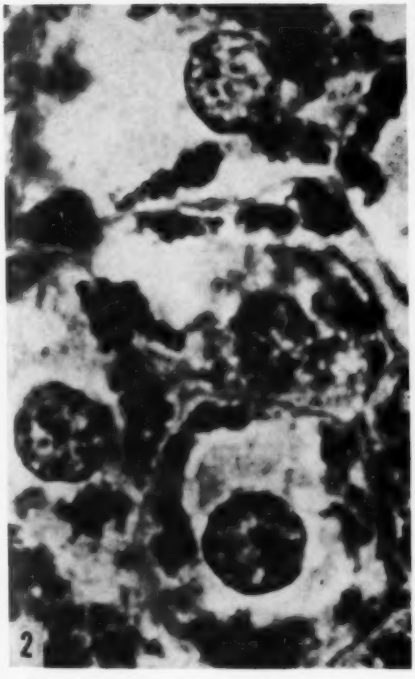
PLATE 1

FIGURE 1.—Liver of a rat after ingestion of *N*-2-fluorenyldiacetamide for 2 weeks. Hepatic cells in the centrolobular zone are vacuolated due to fatty change. Hematoxylin and eosin. × 290

FIGURE 2.—Liver of a rat fed carcinogen for 2 weeks. *Note* aggregates of cytoplasmic basophilic material near the cell membrane. Masson's trichrome stain. × 1,750

FIGURE 3.—Liver of the rat shown in figure 2. *Note* linear aggregates of cytoplasmic basophilic material near the sinusoidal borders of cords of hepatic cells. Hematoxylin-eosin-azure. × 915





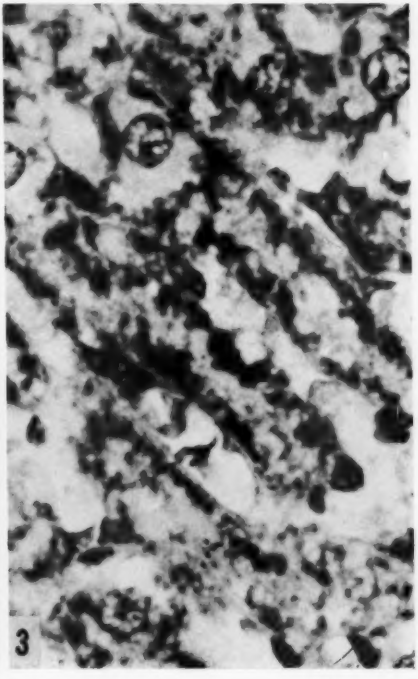


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PLATE 2

FIGURE 4.—Liver of a rat fed carcinogen for 4 weeks. Two cytoplasmic hyaline bodies may be seen. One, *lower-right* field, is crescent-shaped; the other, *upper-left* field, is also crescent-shaped but is contained within the cytoplasm of a binucleated hepatic cell. These structures seemed identical in animals at both 2 and 4 weeks. Only one photograph is presented. Masson's trichrome stain. × 1,500

FIGURE 5.—Liver of a rat fed carcinogen for 2 weeks. A few pseudotubules appear in the periportal space, near the portal vein. Hematoxylin and eosin. × 380

FIGURE 6.—Section of liver from rat described in figure 4, illustrating the radially arranged pseudotubules about the portal vein. Hematoxylin and eosin. × 350

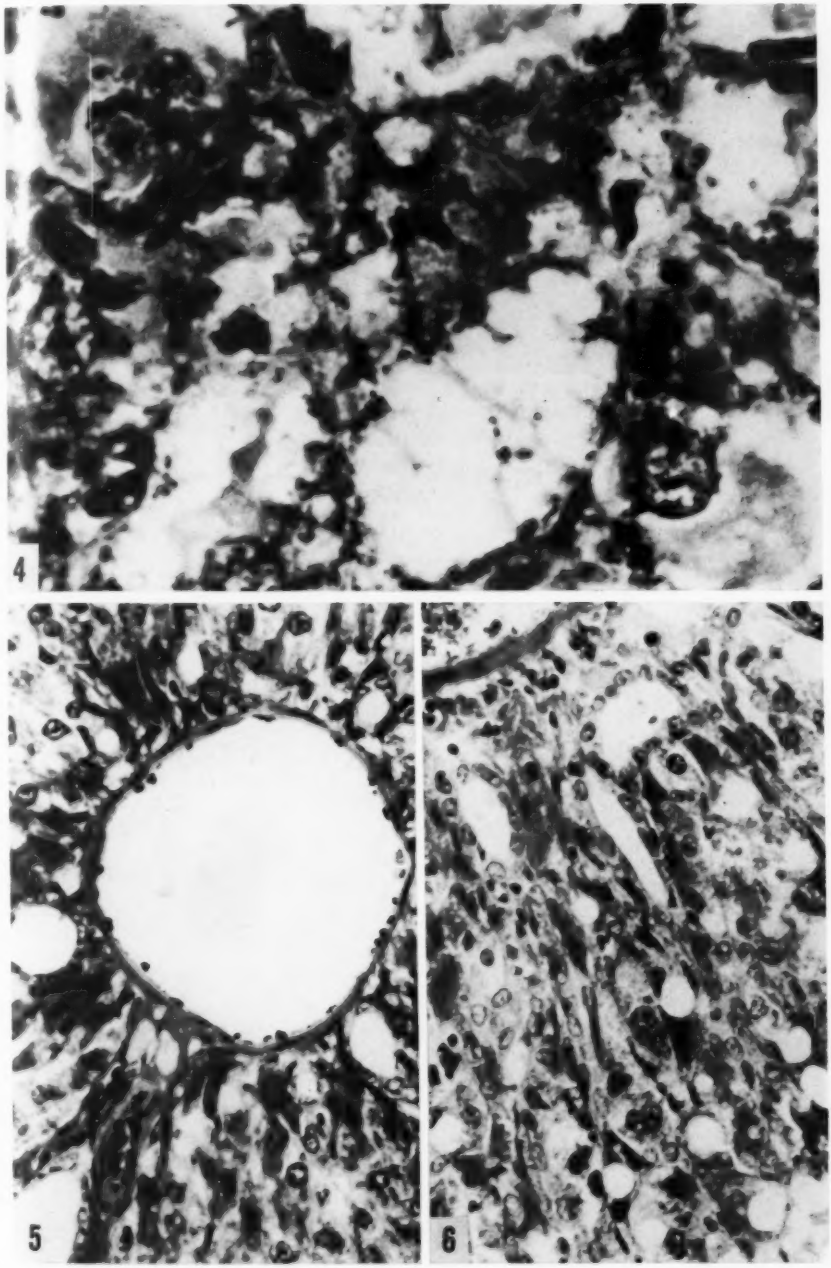
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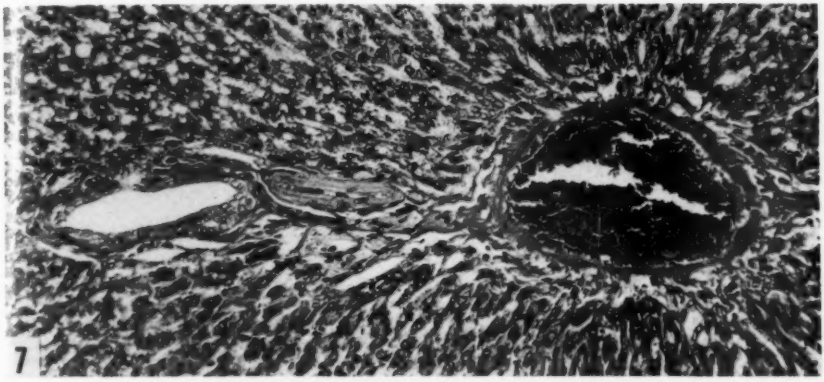
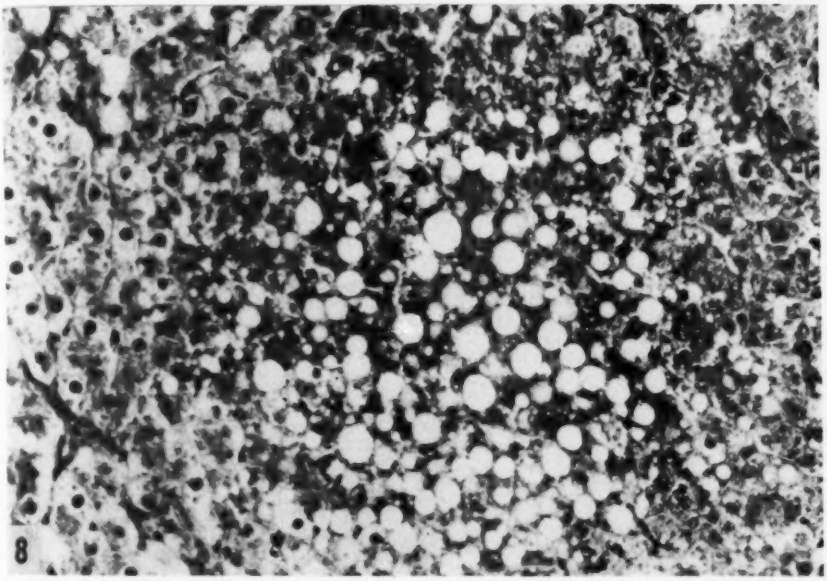
PLATE 3

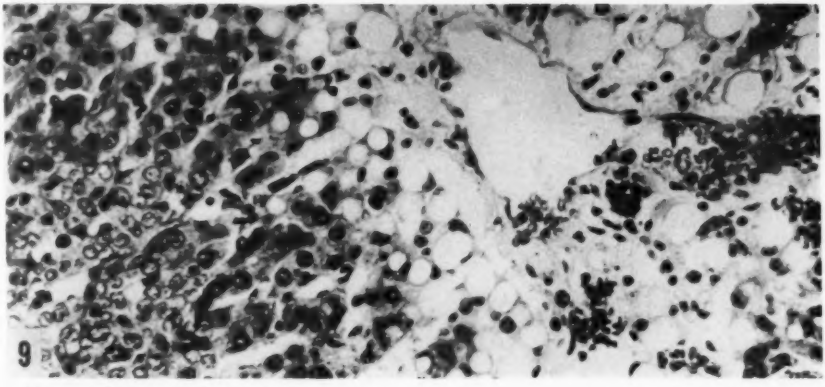
FIGURE 7.—Section of liver from rat fed carcinogen for 2 weeks, picturing the thickening of the walls of the portal vein and its satellite vessels. Hematoxylin and eosin. × 180

FIGURE 8.—Hepatic tissue from rat fed carcinogen for 4 weeks. In *center* of field is a poorly circumscribed area of hepatic-like cells that are more basophilic than the surrounding hepatic tissue and are extensively vacuolated. Hematoxylin and eosin. × 260

FIGURE 9.—Hepatic tissue from animal fed carcinogen for 6 weeks. *Note* extensive vacuolation about the central vein, the midzone of hepatic cells, and the peripheral zone of bile ducts. Hematoxylin and eosin. × 290





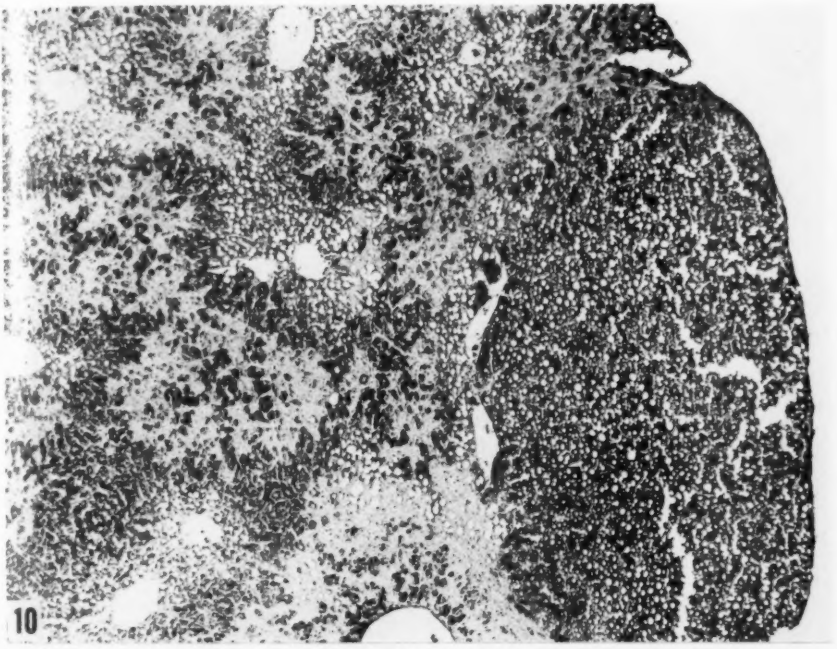


447911—58——4

PLATE 4

FIGURE 10.—Large nodule of hepatic-like cells in a 6-week rat appears along the free margin of the liver, *to right*. Nodule is composed of hepatic-like cells, which stain intensely basophilic. Pale areas represent areas of bile-duct proliferation. Vacuolated areas surround the central veins. Midzones of the lobules stain intensely basophilic. Phosphotungstic acid-hematoxylin. × 65

FIGURE 11.—Section of hepatic tissue from rat receiving diet #284 for 6 weeks, illustrating marked pseudotubule formations in the *extreme right* field and a hyperplastic liver nodule occupying the remainder of the field. Hematoxylin and eosin. × 350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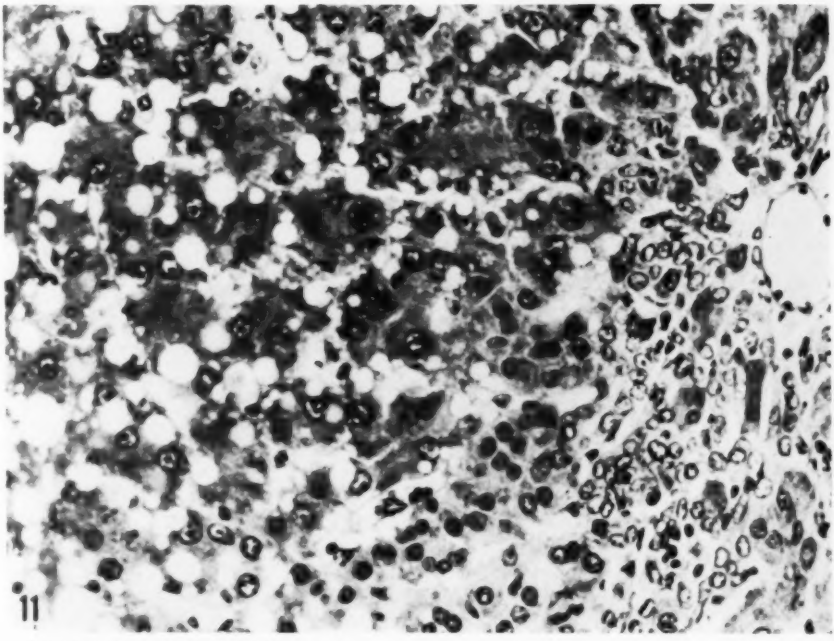
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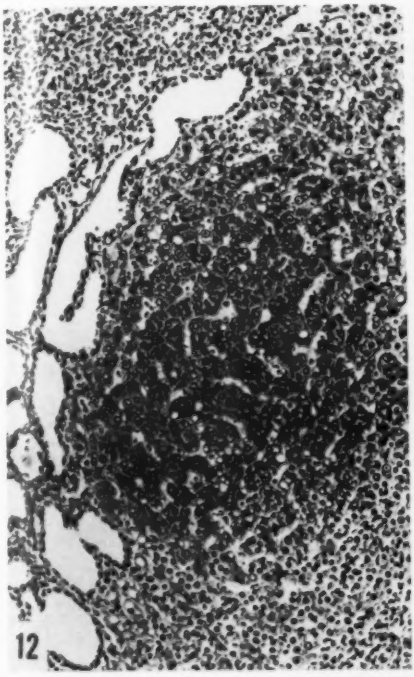
PLATE 5

FIGURE 12.—Section of liver from rat receiving diet #284 for 6 weeks, illustrating a hyperplastic liver nodule and a multilocular cystic area. Wilder's reticulum stain. × 135

FIGURE 13.—Carcinoma occurring in liver of rat receiving the carcinogen for 8 weeks. Hematoxylin and eosin. × 100

FIGURE 14.—Liver of rat fed carcinogen for 8 weeks. Area pictured surrounded a carcinoma, which is not shown. The area contains pseudotubules with associated connective tissue, a few inflammatory cells, and some histiocytes containing ceroid pigment. Residual hepatic cells are divided into small groups by the pseudotubules. Hematoxylin and eosin. × 915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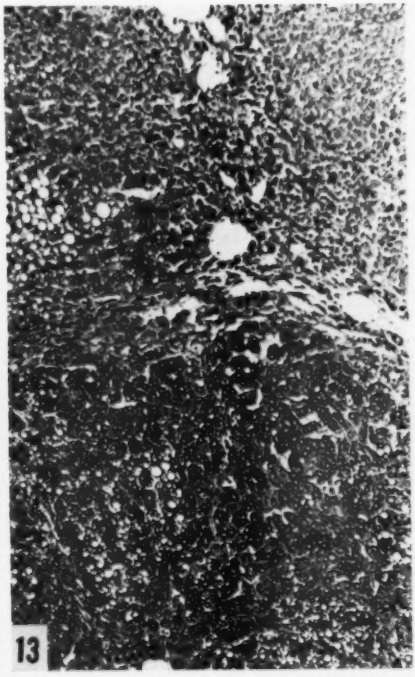
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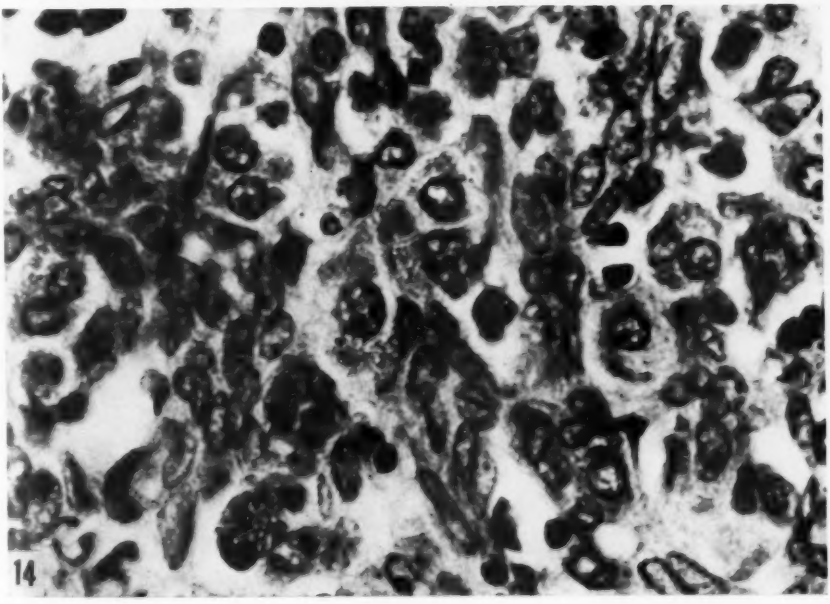
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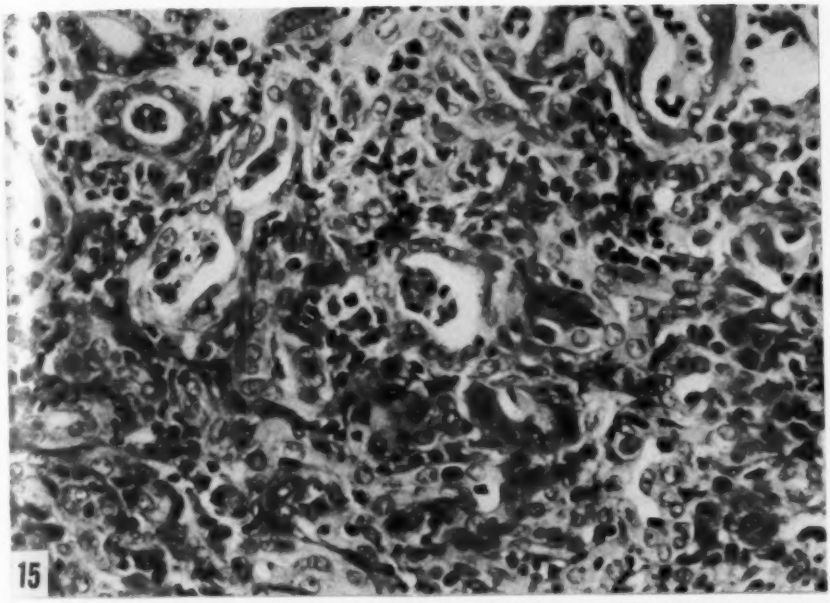
PLATE 6

FIGURE 15.—Area of adenofibrosis (cholangiofibrosis) found in the liver of rat fed carcinogen for 8 weeks. Hematoxylin and eosin. × 340

FIGURE 16.—Liver of rat on diet #285 for 2 weeks. *Note* peripheral lobular vacuolation due to fatty change. Hematoxylin and eosin. × 200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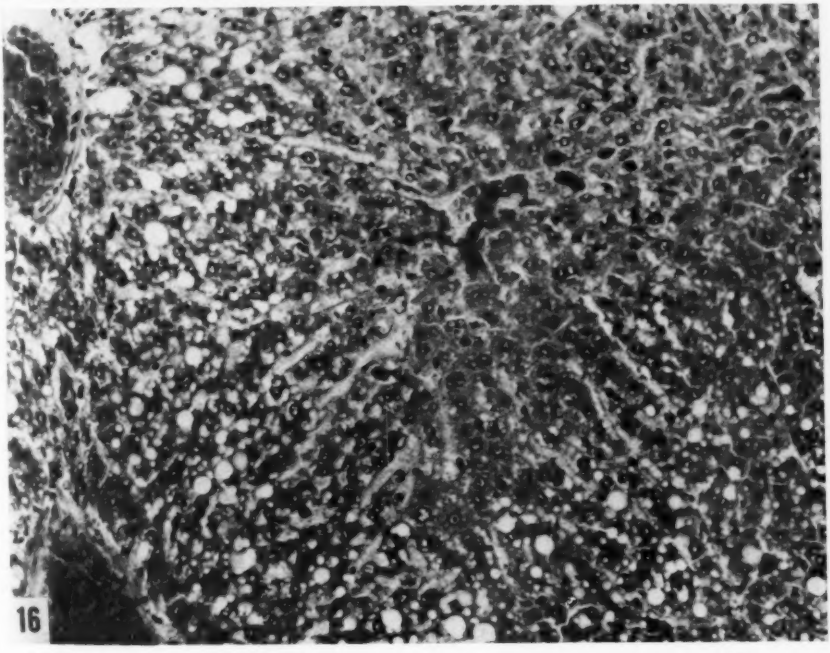
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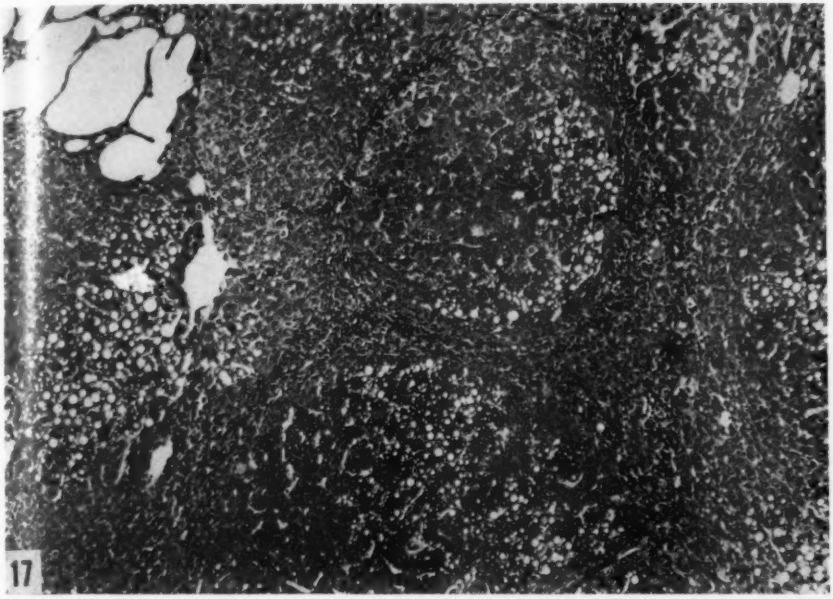
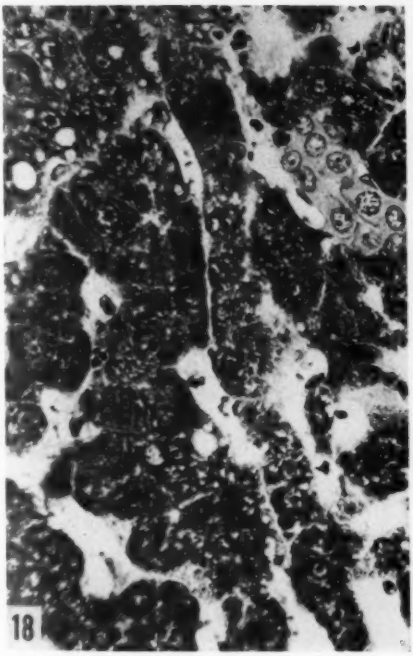
PLATE 7

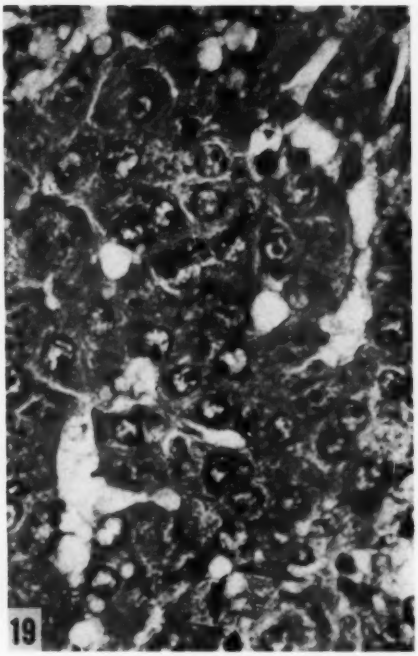
FIGURE 17.—Two early carcinomas are pictured in liver from rat fed diet containing carcinogen for 10 weeks. A multilocular cystic area appears in the *upper-left corner*. Hematoxylin and eosin. × 100

FIGURE 18.—Carcinoma occurring in the liver of rat fed carcinogen for 10 weeks. *Note* pale-staining tubular structure that is in continuity with hepatic-like cells. Hematoxylin and eosin. × 420

FIGURE 19.—Carcinoma occurring in liver of rat fed carcinogen for 10 weeks. *Note* pseudoacinar structure of the hepatic-like cells, variations in nuclear size, and several mitotic figures. Hematoxylin and eosin. × 600







447911—58——5

Polycyclic Aromatic Hydrocarbons in Coffee Soots [1]

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National Cancer Institute,[4] *Bethesda, Maryland*

Roffo (*1–3*) reported that a tar obtained from coffee beans by destructive distillation at 120 to 350° C. showed strong carcinogenic properties when applied to the skin of rabbits or when given orally to rats. He claimed to have demonstrated benzo[*a*]pyrene (1,2-benzpyrene) in the coffee tar. The validity of his observations, however, is open to doubt, because the spectral data presented differ considerably from those accepted as characteristic for benzo[*a*]pyrene. Widmark (*4*), who subsequently tested a petroleum ether extract of roasted coffee for carcinogenic properties by painting it on the skin of mice, could not produce any tumors at the site of application, although a few mammary tumors were observed in some of the animals. Eichler and Vollmer (*5*) more recently reported that they could not observe any carcinogenic properties of coffee tar in an experiment like that of Roffo (*1–3*). Therefore, the problem does not seem to have been adequately investigated.

The present study of coffee soots formed during the roasting of coffee beans was stimulated by the fact that the atmosphere of an American city having excessive morbidity and mortality rates of lung and larynx cancers (*6*) is polluted with effluents from coffee-roasting plants. It was of interest, therefore, to determine whether or not the soots from the roasting processes deposited in the smokestacks contained polycyclic hydrocarbons, in particular those of a known carcinogenic type.

Experimental Procedures

Coffee soots.—Two different coffee soots obtained from two coffee-roasting plants, located in a city in the Southern United States, were analyzed in the present study. The coffee soots were collected from the bottom of the metal stacks attached to the roasters. One of the soots was formed by the so-called "direct" roasting operation in which a gas flame is projected under pressure through the center of the roasting drum

[1] Received for publication June 19, 1957.
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[3] The authors wish to express their appreciation to Dr. Charles H. Grogan and Dr. H. J. Cahnmann of the National Institutes of Health for their helpful advice.
[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

containing the green beans and the drum rotated around the flame. The coffee beans under these conditions are in direct contact with the flame. The other one was produced by the "indirect" roasting process. In this operation coffee beans are roasted by heat from a gas flame applied to the outside of a rotating kiln. The two soots resulting from these processes hereafter will be designated "direct" and "indirect" coffee soot, respectively.

The temperature attained during the roasting was reported to vary from 165 to 250° C., depending upon the type of roast (light or dark), in both the indirect and direct roasting processes. It was also stated that the tip of the flame may reach a temperature as high as 540° C. and that in the indirect roasting procedure the outside of the kiln in contact with the gas flame may be heated to approximately 480 to 540° C. Therefore it is likely, especially in the direct roasting operation, that some of the coffee beans may be burned during both kinds of operations. The two types of coffee soots analyzed were blackish-gray and consisted of fragile, flat, and somewhat greasy, lumps of various sizes. They were ground to a very fine powder with a micropulverizer, the inside of which had been cleaned with benzene before use. One batch of each type of coffee soot as well as batches of green coffee beans used in the two plants were analyzed.

Reagents

Benzene.—Commercial grade, J. T. Baker Chemical Co.
Acetone.—Reagent grade, Fisher Scientific Co., and Merck & Co., Inc.
Dichloromethane.—Commercial grade, Phillips Petroleum Co.
Chloroform.—U. S. P. grade, Mallinckrodt Chemical Works.
Cyclohexane.—Commercial grade, Phillips Petroleum Co.
n-Pentane.—Commercial grade, Phillips Petroleum Co.
Ethanol.—Pure grade (95%).
Methanol.—Pure grade (95%).
Iso-octane.—Spectro grade, Phillips Petroleum Co.

All these solvents except *iso*-octane showed a strong bluish-white fluorescence when exposed to ultraviolet light. They were purified as follows: dichloromethane, chloroform, methanol, and ethanol by repeated distillations; benzene, acetone, *n*-pentane, and cyclohexane by shaking with activated carbon followed by repeated distillations until the residual liquid in the distillation flask became nonfluorescent. In all cases the first and last approximate 5 percent of the distillate was discarded. The boiling ranges of the solvents thus purified were 79.5 to 81.5° C. for benzene, 80.3 to 81.0° C. for cyclohexane, 36 to 41° C. for *n*-pentane, 56.0 to 56.3° C. for acetone, 59.5 to 61.0° C. for chloroform, 39.7 to 41.0° C. for dichloromethane, 77.5 to 78.8° C. for ethanol, and 65.0 to 66.0° C. for methanol.

Caffeine.—Reagent grade, Merck & Co., Inc.
Sulfuric acid.—Reagent grade, Allied Chemical and Dye Corp.
Sodium sulfate.—Reagent grade, Merck & Co., Inc.
Celite.—Analytical, Johns-Manville; purified by washing with acetone.
Carbon, decolorizing.—Allied Chemical and Dye Corp.; activated by heating at 170° C. overnight.

Silica gel.—Grade 922, Davison Chemical Corp.; mesh size "through 200," reactivated by heating at 170° C. overnight. From this reactivated silica gel various activity grades were prepared by adding from 3 to 12 percent water (*7*).

Alumina.—Grade Al-0102 P., Harshaw Scientific; reactivated by heating at 170° C. overnight. Alumina containing 3 and 6 percent water was prepared by adding appropriate amounts of water to the reactivated alumina.

Florisil.—Floridin Co., mesh size "through 200."

Nitrogen, water-pumped.—Southern Oxygen Co. Trace amounts of oxygen contained in the nitrogen were removed by passing the gas through a bottle containing vanadous sulfate solution (*8*). The nitrogen then was dried by passing it through a column of activated alumina.

Reference compounds.—Phenanthrene, pyrene, fluoranthene, benz[*a*]anthracene, chrysene, perylene, benzo[*e*]pyrene, benzo[*a*]pyrene, benz[*k*]fluoranthene, benz[*ghi*]perylene, anthanthrene, and coronene were purified chromatographically.

Instruments

Spectrophotometer.—Cary recording spectrophotometer, model 11 MS, Applied Physics Corp.

Ultraviolet light source.—A light beam from a General Electric H 100-A4 mercury arc bulb was passed through a quartz condensing lens and a Corning filter #5840.

Fraction collector.—Technicon T/F fraction collector, Technicon Chromatography Corp.

Micropulverizer.—Type CF, Pulverizing Machinery Co.

Glassware.—All glassware was checked before use with ultraviolet light for fluorescence caused by contaminants.

Illumination of room.—General Electric gold fluorescent bulbs were used for illumination of the laboratory in which all experiments were carried out.

Analysis

The experimental procedures consisted of a series of extractions with suitable solvents, a preliminary chromatographic fractionation of the extract to separate polycyclic hydrocarbons present in the extract from caffeine and other more polar components, and successive chromatographic fractionations of the eluate fractions until ultimately definitive ultraviolet absorption spectra were obtained. Before the principal analyses were undertaken, preliminary experiments were carried out.

Preliminary Experiments

A) Caffeine content of coffee soots.—According to the information furnished by the manufacturers, the coffee soots contain considerable amounts of caffeine. For achieving an effective extraction of polycyclic hydrocarbons present in the coffee soots its approximate concentration was determined. Two samples of the indirect coffee soot and one sample of the direct coffee soot were analyzed for their caffeine concentration by the Power-Chesnut method (*9*). The values 16.7 and 17.5 percent (average 17.1 percent) by weight for the former and 16.2 percent by weight for the latter were obtained.

B) Separation of benzo[a]*pyrene from caffeine-benzo*[a]*pyrene mixture.*—When benzo[*a*]pyrene was added to caffeine and the mixture placed on a

column of activated alumina, an almost complete separation of the two chemicals was obtained in the benzene and acetone eluates, respectively. The benzene eluate contained up to 99.8 percent of the benzo[*a*]pyrene added, while in the acetone eluate the entire amount of caffeine added was recovered.

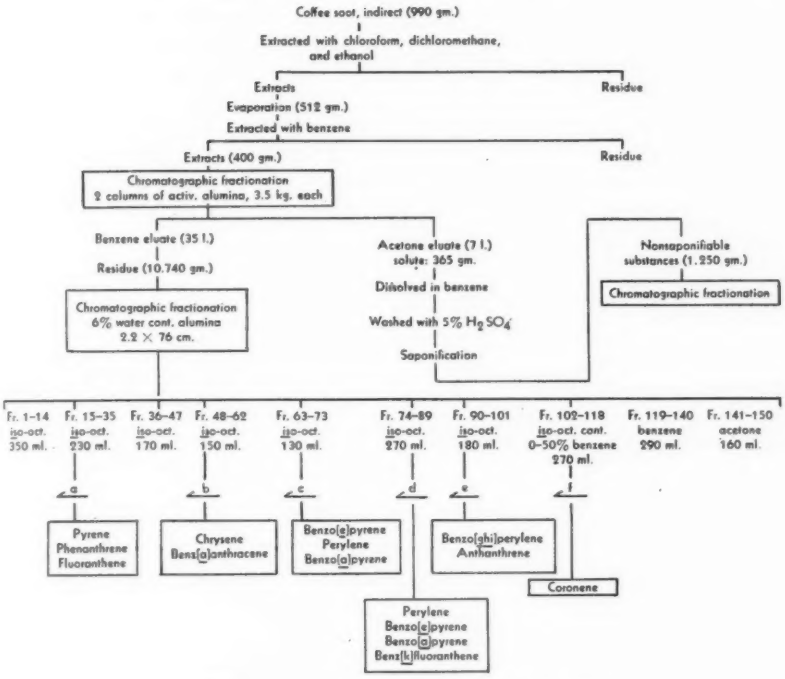
C) *Extraction and chromatographic fractionation.*—In order to determine the most suitable solvent and solvent mixture for an effective extraction of polycyclic hydrocarbons and the chromatographic behavior of such extracts, several preliminary experiments were performed. Their results showed that all the polycyclic hydrocarbons present in the coffee soot could be extracted with acetone and chromatographically separated from caffeine using activated alumina as adsorbent and benzene as eluent. The benzene eluates containing these hydrocarbons could be further fractionated into various components by successive chromatographic separations.

D) *Recovery of benzo*[a]*pyrene.*—The polycyclic aromatic hydrocarbons present in the coffee soot were found only in the benzene eluate fractions and not in the acetone eluate fractions of the acetone extract of the coffee soot. The clear-cut separation of polycyclic hydrocarbons from the crude acetone extract in the benzene fraction was confirmed by adding benzo[*a*]pyrene to the acetone extract and then using the same procedures of separation employed in the separation of polycyclic aromatic hydrocarbons present in coffee soot. Recoveries of benzo[*a*]pyrene from chromatography of the nonsaponifiable residue were 91.0 and 79.5 percent. It is thus seen that no serious losses will result even if benzo[*a*]pyrene is present in the acetone eluate fractions.

Principal Experiment

Coffee soots.—As mentioned before, two different types of coffee soot were analyzed. Because the analytical procedures were alike, the analysis of the indirect coffee soot only will be described here. Its flow sheet is shown in text-figure 1. Nine hundred and ninety gm. of the ground indirect coffee soot was extracted with 3 l. of chloroform at 40° C. in a nitrogen atmosphere for 6 hours in a flask of 4 l. capacity that was connected with a reflux condenser. The extract was filtered through a Büchner funnel and condensed to approximately 500 ml. by distillation under reduced pressure. This distillation was difficult owing to large amounts of caffeine. Therefore, the condensate was spread and evaporated with activated alumina in a desiccator at 25° C., at about 30 mm. Hg for 15 days. The residue weighed 294 gm.

The remaining coffee soot was further extracted 6 times, each with 3 l. of dichloromethane at 40° C. in a nitrogen atmosphere for 5 to 6 hours. The extracts were evaporated by distillation. The combined residue weighed 187 gm. In the preliminary experiment, acetone was used for the extraction, but in this experiment chloroform and dichloromethane were subsitituted, since caffeine is more soluble in these agents. These chlorinated solvents also have lower boiling points than acetone, which



TEXT-FIGURE 1.—Fractionation of coffee soot.

∠a. Silica gel containing 6 percent H_2O, 6 × 630 mm., *n*-pentane containing 0 to 20 percent benzene; alumina containing 3 percent H_2O, 6 × 400 mm., *n*-pentane.

∠b. Silica gel containing 6 percent H_2O, 6 × 460 mm., *n*-pentane containing 0 to 30 percent benzene, silica gel containing 3 percent H_2O, 6 × 450 mm., cyclohexane containing 0 to 15 percent benzene; florisil, 6 × 350 mm., cyclohexane.

∠c. Alumina containing 3 percent H_2O, 7 × 440 mm., *n*-pentane containing 0 to 15 percent benzene; alumina containing 3 percent H_2O, 6 × 520 mm., cyclohexane containing 0 to 10 percent benzene; florisil 6 × 400 mm., *iso*-octane containing 0 to 10 percent benzene.

∠d. Alumina containing 3 percent H_2O, 7 × 430 mm., *n*-pentane containing 0 to 15 percent benzene; alumina containing 3 percent H_2O, 6 × 480 mm., cyclohexane containing 0 to 10 percent benzene.

∠e. Alumina containing 3 percent H_2O, 7 × 400 mm., *n*-pentane containing 0 to 15 percent benzene; alumina containing 3 percent H_2O, 6 × 300 mm., cyclohexane containing 0 to 10 percent benzene.

∠f. Alumina containing 6 percent H_2O, 6 × 440 mm., *n*-pentane containing 0 to 20 percent benzene.

facilitates the evaporation of the extract. After extraction with dichloromethane, the coffee soot was again extracted 3 times, each time with 3 l. of ethanol at 50° C. for 6 hours. Upon evaporation of the extracts 31 gm. of a tarry, brown residue were obtained. The distillate of these extracts sometimes fluoresced weakly in ultraviolet light. In such cases

the distillate was again distilled and its residue was combined with the original residue. All the residues thus obtained were combined making a total of 512 gm. and representing 51.7 percent of the coffee soot. They were then repeatedly extracted with warm benzene. The benzene extract (total volume 12 l., solute 400 gm.) was divided into two equal portions. Each portion was placed on a column of 3.5 kg. of activated alumina (diameter=15 cm. at top, 8 cm. at bottom, and height, 28 cm.) (chromatography 1). The column was developed and eluted with benzene without pressure being applied (flow rate about 14 ml. per minute).

The observations of the column, when developed with about 8 l. of benzene, are shown in table 1. Zone A hardly moved with benzene and zones B (caffeine zone) and C did so very slowly, while zone D was quickly eluted with benzene. When zone C approached the bottom of the column, benzene was replaced by acetone. Zones C and B and some components of zone A were eluted giving 7 l. of acetone eluate fractions and leaving a brown-colored substance on the column. All the benzene eluate fractions (35 l.) were carefully evaporated by distillation under reduced pressure.

TABLE 1.—*Observations of the column when developed with about 8 l. of benzene (chromatography 1)*

Zone	Observations	
7 cm. from top	Dark-brown-colored	A
3 cm	Gray-colored, grayish-white fluorescent	
0.5 cm	Yellowish-gray-colored, dark gray fluorescent	
0.5 cm	Not colored, bluish-white fluorescent	
6 cm	Faintly yellow-colored, faintly whitish fluorescent	B
0.3 cm	Yellow-colored, weakly yellow fluorescent	C
0.3 cm	Not colored, bluish-white fluorescent	
10 cm. at bottom	Not colored, faintly whitish-blue fluorescent	D

The distillate was checked in ultraviolet light. A slightly yellow-colored residue (10.740 gm.) was obtained. It was dissolved in about 80 ml. of *iso*-octane and chromatographed on a long column of alumina containing 6 percent water (300 gm., 22 × 760 mm.), using *iso*-octane as developer (chromatography 2). No pressure was applied on the column (flow rate about 0.8 ml. per minute). Table 2 represents the appearance of the column developed with about 300 ml. of *iso*-octane. As the

TABLE 2.—*Observations of the column when developed with about 200 ml. of* iso*-octane (chromatography 2)*

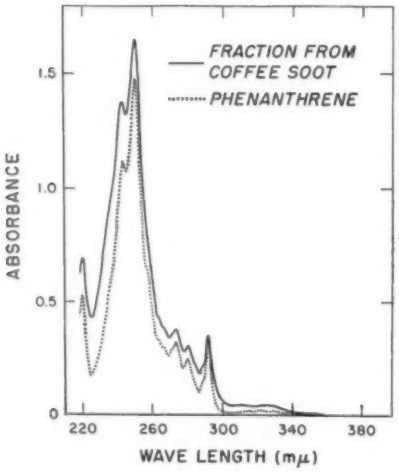
Zone	Observations	
15 cm. from top	Light-brown-colored, brownish-gray fluorescent	
0.4 cm	Yellow-colored, yellow fluorescent	
4 cm	Faintly light-yellow-colored, weakly blue fluorescent	(I)
13 cm	Faintly yellow-colored, greenish-blue fluorescent	(II)
4 cm	Not colored, purplish-blue fluorescent	(III)
16 cm	Not colored, bluish-white strongly fluorescent	
18 cm	Not colored, strongly blue fluorescent	(IV)
6 cm. bottom	Not colored, faintly blue fluorescent	

development with *iso*-octane was continued, zone II in table 2 became wider and strongly purplish-blue fluorescent. Zone III also increased its intensity of fluorescence. Approximately 1,500 ml. of *iso*-octane were used and 110 fractions, each 8 to 20 ml. in volume depending on the width of the zones collected, were obtained.

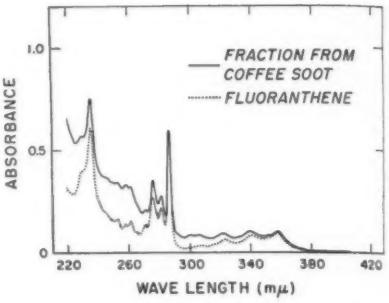
The absorption spectra of these fractions suggested that zone IV contained phenanthrene, pyrene, and fluoranthene; zone III, benz[*a*]-anthracene, chrysene, and perylene; zone II, perylene, benzo[*e*]pyrene, benzo[*a*]pyrene, benz[*k*]fluoranthene, and zone I, benz[*ghi*]perylene and traces of anthanthrene and coronene. The fractions that showed similar absorption spectra were combined and subjected to further chromatographic fractionations on various adsorbents until all absorption peaks characteristic for a certain hydrocarbon were clearly observed.

In early experiments, repeated chromatographic fractionation was done on relatively short columns (7 to 15 cm. in most cases) when a mixture consisting of 2 or 3 components was to be fractionated, and slight pressure was sometimes applied on the columns in order to maintain a moderate flow rate, *i.e.*, 5 to 10 mm. per minute. However, in this experiment, fairly long columns (30 to 60 cm.) were often used and no pressure was applied, so that the flow rate was approximately 1 mm. or less per minute. These slow chromatographies performed on long columns resulted in excellent separation of the various components. Two or more chromatographies could be carried out at the same time using a fraction collector. As to the dimensions of the columns, type of adsorbents, and solvents used, reference is made to the legend of text-figure 1. The complete separation of benz[*a*]anthracene and chrysene was extremely difficult. Various combinations of adsorbents and developers were tried for this purpose. Chrysene could almost completely and relatively easily be separated from benz[*a*]anthracene, probably because the concentration of the former was considerably higher than the latter (text-fig. 5). However, purification of benz[*a*]anthracene was only partially achieved as shown in text-figure 6. Similar difficulties were encountered in the separation of benz[*ghi*]perylene and anthanthrene, where a trace amount of the latter compound could not be eliminated from the former (text-fig. 11).

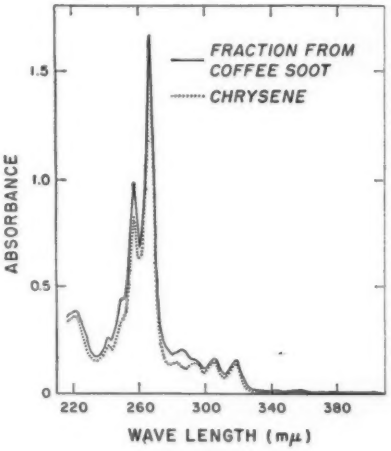
The acetone eluates of chromatography 1 were combined and carefully condensed by distillation under reduced pressure. Great difficulties again were encountered in performing their condensation due to an uneven boiling of the solution caused by the large amount of caffeine crystals. Therefore, approximately 800 ml. of the condensed eluate fraction was spread and evaporated with activated alumina in a desiccator at 25° C. in about 30 mm. Hg for 18 days. The alumina was frequently exchanged with freshly activated alumina. In this manner, 365 gm. of a slightly greasy light-brown-colored residue was obtained. It was dissolved in about 12 l. of benzene. Portions of 2 l. each of this benzene solution were washed 3 times with 1 l. of 5 percent H_2SO_4. The benzene solution thus treated was washed 3 times with 3 l. of water each, then desiccated over sodium sulfate, and condensed to 200 ml. The condensed benzene solution was saponified



TEXT-FIGURE 2.



TEXT-FIGURE 4.



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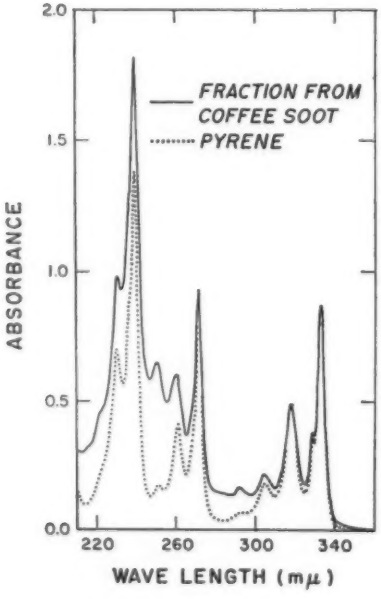
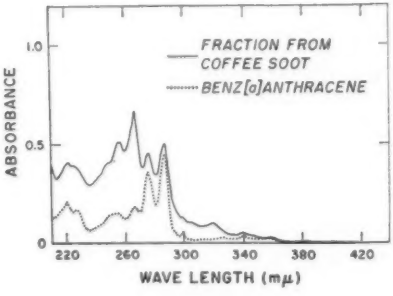


TEXT-FIGURE 3.



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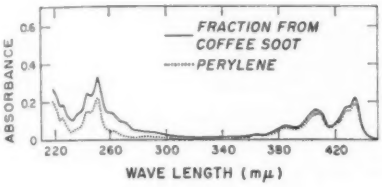
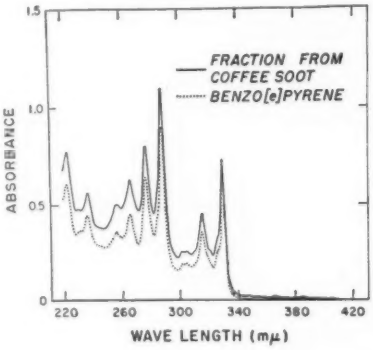
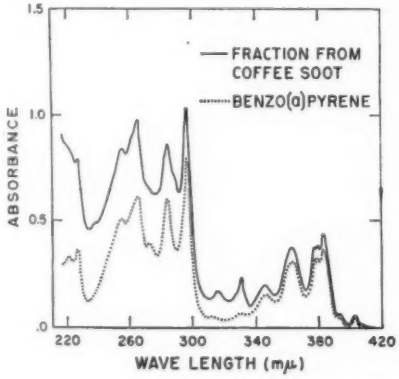


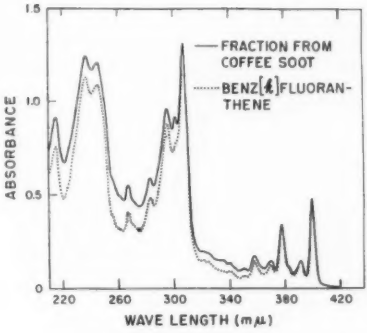
TEXT-FIGURE 7.



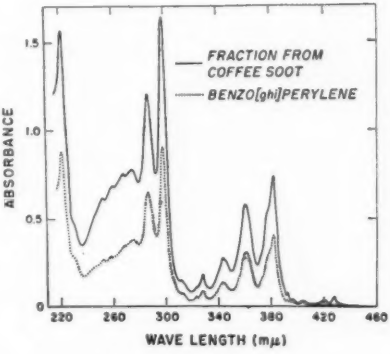
TEXT-FIGURE 8.



TEXT-FIGURE 9.



TEXT-FIGURE 10.



TEXT-FIGURE 11.

with 100 ml. of 10 percent KOH in methanol at 25° C. for 4 hours, and then washed 4 times with 1 l. of water each, followed by drying over sodium sulfate and by evaporation. The residue (1.250 gm.) was dissolved in cyclohexane containing 50 percent benzene and chromatographed on a column of 3 percent water containing alumina (7 × 200 mm.) using the same solvent mixture as developer. The absorption spectra of the eluate fractions did not exhibit structural details typical for polycyclic hydrocarbons. In view of the results of the preliminary experiments, the benzene-insoluble, waxy material derived from the initial chloroform, dichloromethane, ethanol extract of the coffee soot was not further analyzed.

The identification of the hydrocarbons was based on a comparison of the spectra obtained with those of authentic samples of pure hydrocarbons determined under identical conditions. For a rough estimate of the amounts of the polycyclic hydrocarbons present in the various eluate fractions, the baseline technique described by Cooper (*10*) was used. Absorption peaks in the higher wavelength region were selected for this determination because in this region there is least interference from background absorption (292 mμ. for phenanthrene; 333 mμ. for pyrene; 287 mμ. for fluoranthene; 319 mμ. for chrysene; 287 mμ. for benz[*a*]anthracene; 434 mμ. for perylene; 330 mμ. for benzo[*e*]pyrene; 383 mμ. for benzo[*a*]-pyrene; 400 mμ. for benz [*k*] fluoranthene; 383 mμ. for benz [*ghi*] perylene).

Green coffee beans.—Two kinds of green coffee beans used in direct and indirect roasting operations were analyzed. Two batches of coffee beans weighing 1,362 gm. and 2,027 gm., respectively, were extracted 3 times with cyclohexane, each time with 2 l. at 25° C. for 30 minutes. The extracts were evaporated under reduced pressure. Brown, oily residues weighing 1.742 and 1.637 gm., respectively, were obtained. These residues were dissolved in cyclohexane and saponified with methanol containing 10 percent KOH at 25° C. for 4 hours. The cyclohexane layers, after being washed with water, were centrifuged at 3,000 r.p.m. to separate and remove large amounts of a cloudy, gelatin-like material. The sediments were repeatedly washed with cyclohexane until the supernatant fluid became nonfluorescent. Thus, a slightly cloudy but not completely transparent cyclohexane extract was obtained that was dried with Na_2SO_4 and condensed by evaporation of the solvent. The residues weighed 1.038 and 0.952 gm., respectively.

Although these residues still contained a relatively large amount of gelatin-like material, they were placed on a chromatographic column of activated alumina containing 30 percent celite. The column was overlaid with a celite layer, 5 cm. in height, in order to prevent clogging by the gelatinous matter. The column was eluted with 300 ml. of benzene. The benzene eluate fractions which contained 0.267 gm. or 0.257 gm. solute, respectively, were further subjected to chromatographic fractionations with a method like that employed in the analysis of coffee soots. The absorption spectra of these chromatographic eluate fractions did not show evidence of the presence of polycyclic hydrocarbons identical with those found in the coffee soots.

Results

The polycyclic aromatic hydrocarbons positively identified and their approximate concentrations are listed in table 3. Besides these compounds, the presence of relatively large amounts of alkyl-substituted naphthalenes, phenanthrenes, pyrenes, and chrysenes was suspected by the observed bathochromic shift of their characteristic absorption peaks. The unequivocal identification of these substituents, however, was not achieved because of sufficient variety of reference compounds for comparison could not be obtained. However, the maxima of spectral absorption for these compounds are as follows:

	Main peaks observed
Alkyl-substituted naphthalenes	*228–230* mμ.
" " phenanthrenes	*253–255*, 275–278; 299–302 mμ.
" " pyrenes	*243*, 275, 335 mμ.
" " chrysenes	259, 260; *269–271*; 321 mμ.

(The numbers italicized are the most prominent peaks for each compound.)

A trace amount of anthanthrene was also found in both direct and indirect coffee soots, while a trace of coronene was demonstrated only in the indirect coffee soot.

TABLE 3.—*Approximate amount of polycyclic aromatic hydrocarbons found in coffee soots*

Hydrocarbons	Direct coffee soot (γ/kg.)	Indirect coffee soot (γ/kg.)
Phenanthrene	130	300
Pyrene	260	720
Fluoranthene	340	1000
Chrysene	530	670
Benz[*a*]anthracene	16	150
Perylene	280	660
Benzo[*e*]pyrene	190	370
Benzo[*a*]pyrene	200	440
Benz[*k*]fluoranthene	70	140
Benzo[*ghi*]perylene	100	140

Discussion

One of the chief technical difficulties that had to be overcome in the analysis of coffee soot was to find a method of eliminating large amounts of caffeine without losing any polycyclic hydrocarbons. Dr. Weil-Malherbe's extensive studies on the solubilization of polycyclic aromatic hydrocarbons by purines (*11*) supplied much helpful information. A simple chromatographic fractionation with activated alumina and benzene as developers, which he used to separate hydrocarbons including benzo[*a*]pyrene and coronene from their molecular complexes with purines, proved to be applicable to the present analysis of the highly complex extracts of coffee soots. It separated effectively not only caffeine but also the other constituents, *i.e.*, sterols, pigments, and acidic and alkaline components. Further chromatographic fractionations of polycyclic hydrocarbons could be performed efficiently with this procedure.

At the present time, the origin of the polycyclic hydrocarbons that were found cannot be established with certainty. Three possibilities had to be considered in this regard: 1) the formation of aromatic hydrocarbons by pyrolysis of the coffee beans; 2) contamination with polycyclic hydrocarbons that are formed in the flame used in the roasting process; and 3) contamination of the beans prior to the roast. The second possibility either may be dismissed completely or is at best only a contributory factor because the hydrocarbons found in the direct coffee soot were also in the indirect coffee soot. In view of the negative results of the analysis of green coffee beans, the third possibility may be excluded. It appears therefore that the production of the polycyclic aromatic hydrocarbons, particularly those with carcinogenic properties found in coffee soot, may be attributed mainly to the pyrolytic action of heat on coffee beans.

A question arises whether the temperatures employed in the roasting processes are sufficiently high for the formation of polycyclic hydrocarbons. There are many studies on the carcinogenicity of pyrolysis products of organic substances and the formation of aromatic hydrocarbons by pyrolysis or incomplete combustion. However, only a few investigations are concerned with the threshold temperature essential for the formation of polycyclic aromatic hydrocarbons, especially those consisting of four or more rings. It seems that threshold temperatures probably vary with, and are dependent upon, the type of substances subjected to pyrolysis, pressure, degree of oxygen supply, and other factors.

Staudinger *et al.* observed that isoprene tar produced at 700° C. consisted mainly of unsaturated hydrocarbons, while that produced at 820° C. consisted of saturated aromatic hydrocarbons (*12*). Kennaway indicated that the tar obtained from isoprene, heated at 820° C. by a method like that of Staudinger *et al.*, possessed strong carcinogenicity for the skin of mice (*13*). Falk *et al.* (*14*) noted that the pyrolysis products of cholesterol obtained at 360° C. contained cholestenone, 3,5-cholestadiene, naphthalene derivatives of cholesterol, and chrysene. Kirby reported the presence of methylcyclopentenophenanthrene in the cholesterol heated to 430° C. (*15*). The noncarcinogenicity of pyrolysis products of cholesterol obtained at 200° C. has been confirmed by several workers (*14*). In view of these studies, the threshold temperature for pyrolytic formation of aromatic hydrocarbons does not seem to lie below 250° C. In the present experiment, the polycyclic aromatic hydrocarbons found were probably formed from the coffee beans during exposure to a temperature of approximately 540° C.—either in contact with the gas flame or with the wall of the kiln. Moreover, it is likely that during the roasting process, at times, much higher temperatures may prevail resulting in the actual burning of the beans.

Because of the greater probability that during the direct roasting operation some of the coffee beans may be burned by direct contact with the gas flame, it was supposed that this method might produce

more polycyclic hydrocarbons than the indirect one. Contrary to expectation, the present investigation showed that the direct coffee soot contained less polycyclic hydrocarbons than the indirect one. However, considerable variations in the relative and absolute amounts of polycyclic hydrocarbons produced seem to exist in different batches of coffee soot. In the preliminary experiment C, pyrene, chrysene, benz[*a*]anthracene, and perylene were found in a sample of 200 gm. from a batch of indirect coffee soot, but no benzo[*a*]pyrene, benzo[*e*]pyrene, benz[*k*]fluoranthene and benzo[*ghi*]perylene were found. These latter compounds, on the other hand, were demonstrated in another batch of indirect coffee soot used in the principal experiment. Failure to demonstrate these compounds in the preliminary experiment C cannot be attributed to an experimental error. It is more likely that variations in the polycyclic hydrocarbon content of different batches are due to fluctuations in the roasting temperatures applied in the production of light- and dark-roasted coffee. Whether there exist any differences in the production of polycyclic hydrocarbons between the indirect and the direct roasting operation remains undetermined until several batches of coffee soots produced under proper test conditions have been analyzed.

Among the polycyclic hydrocarbons positively identified, only benzo[*a*]pyrene has been proved to be a potent carcinogen. Only weak or questionable carcinogenic potency has been reported for some of the other components identified (benz[*a*]anthracene, benzo[*e*]pyrene, and benzo[*ghi*]perylene) (*16–18*). Benz[*k*]fluoranthene has not been adequately evaluated in this respect, but it should be thoroughly investigated for possible carcinogenic properties because of its wide distribution in air pollutants (*19, 20*) and also in oysters, which were collected in a harbor area polluted with ship fuel oil (*21*). A bioassay of this compound has been started in our laboratory.

The concentration of benzo[*a*]pyrene in the coffee soots does not seem high. As shown in table 4, it is much lower than that present in wood and coal soots which are carcinogens for animals and man. Nevertheless, the possibility exists that coffee soots may be a factor in human carcinogenesis. Their potential significance in this respect is suggested by the following considerations: 1) Workers in coffee-roasting plants are exposed to this special kind of soot by inhalation, ingestion, and skin contact.

TABLE 4.—*Benzo*[a]*pyrene concentrations in pyrolysis products*

Source	Benzo[*a*]pyrene (*γ/100 gm.*)
Soot, domestic (Gouldon and Tipler, *23*)	30,000
Soot, coal (Kuratsune, *24*)	1,200; 5,600
Soot, wood (Kuratsune, *24*)	170–3,600
Charred matter from biscuit (Kuratsune, *24*)	7.2; 1.1
Smoke, cigarette (Cooper and Lindsey, *16*)	0.91/100 gm. of cigarette, burnt
Smoke, cigarette paper (Cooper and Lindsey, *14*)	4.0/100 gm. of paper, burnt
Smoke, cigarette paper (Cardon *et al.*, *25*)	61/100 gm. of paper burnt
Smoke, cigarette (Cardon *et al.*, *25*)	8–12/100 gm. of cigarette, burnt
Smoke, cigar (Cardon *et al.*, *25*)	11–70/100 gm. of cigar, burnt
Coffee soot (Kuratsune and Hueper)	20; 44

2) Commercial products of roasted coffee are probably contaminated with coffee soot. 3) If benzo[*a*]pyrene exists in roasted coffee, it will easily be transferred into coffee by infusion through forming a water-soluble molecular complex with caffeine (*11*). 4) Coffee soots discharged from coffee-roasting plants pollute the atmosphere in the vicinity of the plants and may be concentrated in the caffeine particles present in the polluted air and demonstrated in air-pollutant samples collected in contaminated areas (*22*). 5) Since the coffee soot is used in the commercial extraction of caffeine employed for medicinal and other purposes, such operations and uses may possibly be associated with cancer hazards.

The introduction of carcinogenic hydrocarbons contained in coffee soot and related products into the human environment doubtlessly contributes a quantitatively undetermined share to the total carcinogenic load of the population. Whether or not any actual and significant cancer hazards are associated with such exposures, sustained because of occupational, dietary, medicinal, or environmental reasons, is at present problematical.

Conclusions

1) The chemical analysis of coffee soots generated during the direct and indirect roasting of coffee beans revealed the presence of 10 identified polycyclic aromatic hydrocarbons. One of them (benzo[*a*]pyrene) is an established carcinogen for experimental animals and is strongly suspected of being carcinogenically active in man. The relative amount of this carcinogen in the coffee soot (about 200 and 440 μg. per kg. of soot) is rather small.

2) Whether or not any actual cancer hazards are associated with sustained exposures to coffee soot and related products because of occupational, dietary, medicinal, or environmental reasons is at present undetermined.

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Effects of 6-Azauracil on Galactose Metabolism in Mice, Dogs, and Man [1, 2]

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The compound 1,2,4-triazine-3,5(2*H*,4*H*)-dione (6-azauracil), known since 1947 (*1*), is one of a group of pyrimidine analogues recently studied for biologic activity by Barlow and Welch (*2*), Falco *et al.* (*3*), and Šorm *et al.* (*4*). It has been demonstrated to have an inhibitory effect on several animal tumors (*4, 5*) and its preliminary evaluation in man has recently been reported (*6*). This compound acts as an antagonist of uridine and uracil in certain microorganisms (*7*).

Uridine diphosphoglucose is an essential compound in the reaction in which the enzyme galactose-phosphate (Gal-1-P) uridyl transferase catalyzes the incorporation of galactose-1-phosphate into a nucleotide form (*8*). This is a necessary step in the transformation of galactose to glucose. Since 6-azauracil is a potential uridine antagonist, it was thought that this drug or a metabolic derivative might interfere with this reaction, and thus, with galactose-glucose transformation (text-fig. 1). To investigate this possibility, galactose metabolism was studied in association with the administration of 6-azauracil: 1) by a modified galactose tolerance test; 2) by direct measurement of Gal-1-P uridyl transferase activity. Studies were performed in mice, dogs, and man.

Studies in Mice

Materials and Methods

In the first experiment, CDBA mice, approximately 4 months of age, were divided into 4 groups. Group 1 consisted of normal mice not given 6-azauracil; group 2, normal mice given 6-azauracil in their drinking

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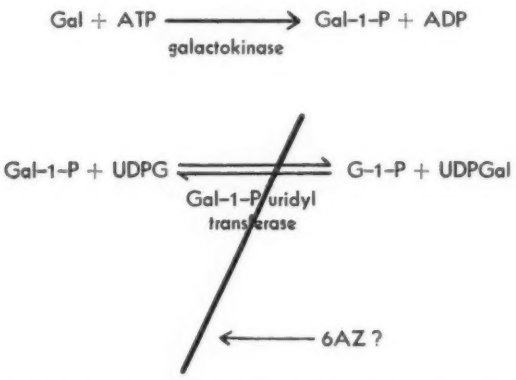
water at a concentration of 5 mg. per ml.[7] Groups 3 and 4 received 0.1 ml. of a 1 : 3 dilution of the ascites form of mouse leukemia L1210 by subcutaneous implantation into the axilla. Group 3 was not given 6-azauracil; group 4 received the drug in the same dosage as group 2, beginning 24 hours after the time of tumor implantation. All animals were fed a standard laboratory diet during the experiment.

Seven days after tumor implantation (6 days of 6-azauracil administration), a modified galactose tolerance test was performed on each animal. The animals were fasted for 2 hours prior to the test, and then 1.5 mg. of galactose per gm. weight of mouse, as a 15 percent aqueous solution, was injected into the tail vein. Ninety minutes after injection, 0.5 ml. of blood was obtained from each mouse by decapitation. Tumor was dissected from the axilla, scraped clean of fat and subcutaneous tissue, and weighed.



TEXT-FIGURE 1.—This figure summarizes the initial steps in galactose-glucose conversion. The heavy oblique line indicates a point in the reaction where 6-azauracil or its derivatives were postulated to inhibit the transformation. Gal = galactose; ATP = adenosinetriphosphate; Gal-1-P = galactose-1-phosphate; ADP = adenosinediphosphate; G-1-P = glucose-1-phosphate; UDPG = uridine diphosphoglucose; UDPGal = uridine diphosphogalactose; 6-AZ = 6-azauracil.

In a previous pilot study with normal mice, blood galactose had fallen to near zero by 90 minutes after injection of galactose. On the basis of this observation, it was felt that a determination of blood galactose at a 90-minute postinjection time point would best demonstrate a retarded disappearance of blood galactose in the experimental animals.

Galactose was measured as the reducing substance remaining in the blood sample after destruction of glucose by glucose oxidase, with a modification of the method of Wyngaarden, Segal, and Foley (*9*). In this procedure, whole blood is hemolyzed in water, and a protein-free filtrate is formed by precipitation with barium hydroxide and zinc sulfate. The filtrate is then incubated with a glucose oxidase enzyme system at

[7] We are indebted to Dr. Arnold Welch, Department of Pharmacology, Yale University School of Medicine, and to the E. R. Squibb Company for generously providing the 6-azauracil used in this study.

37° C. in an oxygen atmosphere for 3 hours. Reducing substance is determined using Nelson's blood glucose procedure (*10*).

In the second experiment, animals were again divided into 4 groups and treated as in the first experiment, but galactose was not administered. Blood analyses were performed in the same way. This was done to ascertain whether non-glucose reducing substances found in the blood were actually due to the galactose administered and not directly related to 6-azauracil administration, tumor, or other factors.

In this experiment, the livers from animals in each group were pooled, and Gal-1-P uridyl transferase activity was measured by the incorporation of radioactive galactose-1-phosphate into nucleotide form, according to the method of Anderson and Kalckar (*11*). The livers were also examined histologically.

Differences between groups, with respect to both blood galactose concentrations and liver weight, were analyzed for statistical significance. Values of probability (P) were determined by Student's t test.

Results

The results of the studies in mice are shown in table 1. Data headed "Experiment 1" are obtained from the first study in which galactose was administered; data headed "Experiment 2" are from the second study in which no exogenous galactose was administered prior to the measurement of non-glucose reducing substance.

In both experiments, tumor weight was less in the treated groups than in the groups not receiving 6-azauracil. This confirms the previous observations on the inhibitory effect of this drug on mouse leukemia L1210 (*5*). A greater degree of tumor inhibition was achieved in the second experiment than in the first, even though apparently identical conditions

TABLE 1.—*Effects of 6-azauracil on liver, tumor, and on galactose metabolism in normal and leukemic mice*

Group	No. animals in group		Average tumor weight (*mg.*)		Average blood galactose (*mg. per 100 ml.*)		Average liver weight (*mg.*)	Transferase in pooled liver*
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 2	Expt. 2
No tumor, No 6-AZ	6	4	—	—	53	0	1350	0.82
No tumor, 6-AZ	6	4	—	—	37	0	1410	0.87
L1210, No 6-AZ	6	4	464	503	132	0	2260	0.89
L1210, 6-AZ	12	4	397	104	58	0	1802	0.96

*Expressed as μM C^{14}-Gal-1-P incorporated into nucleotide per gm. liver per hour.

were in effect. This degree of variability in inhibition with 6-azauracil against L1210 has been observed before (*12*).

In experiment 1, the blood galactose concentrations 90 minutes after galactose injection had fallen to below 60 mg. per 100 ml. in all groups except the L1210 inoculated mice that had not received 6-azauracil. These animals showed an average blood galactose concentration of 132 mg. per 100 ml. This difference was significant, with a value of $P<0.01$. In the non-tumor-bearing animals, the groups treated with 6-azauracil did not, contrary to original expectations, show an elevated blood-galactose concentration, but indeed had a slightly lower concentration than did the control group. However, this latter difference was not significant, P being greater than 0.05.

In the second experiment, where no exogenous galactose was administered, all groups had zero blood concentrations of non-glucose reducing substance. This observation indicates that the elevated concentration seen in the untreated tumor group in experiment 1 resulted from an impaired metabolism of the administered galactose.

Liver weights were higher in the two tumor-bearing groups than in the non-tumor-bearing groups. Although the weight of liver was slightly lower in the tumor-bearing animals treated with 6-azauracil than in those not so treated, this difference was not significant at a 0.05 level of probability.

Histologic examination of the livers of L1210 untreated animals showed the livers to have a marked leukemic infiltration. There was striking compression of liver cells and an increase in sudanophilic staining material. Very little normal hepatic parenchyma could be seen. In the leukemic animals receiving 6-azauracil, there was less severe infiltration. Some of the cellular infiltrate was thought to represent sites of extramedullary hematopoiesis. The normal animals treated with 6-azauracil had normal hepatic histology.

Despite the differences in weight and histology in the livers in the four groups of animals, Gal-1-P uridyl transferase activity per gram weight of liver was approximately the same in all groups, and there was no significant change in the enzymatic activity as a result of the drug administration. The impaired galactose metabolism in the leukemic mice not treated with 6-azauracil was not correlated with a defect in transferase activity.

Studies in Dogs

Two dogs received large oral doses of 6-azauracil (100 mg. per kg. every 8 hours for 3 days followed by 150 mg. per kg. every 8 hours on the fourth day). Blood Gal-1-P uridyl transferase activity was determined before and at the end of the period of treatment with 6-azauracil. The assay used was similar to that described for liver transferase.

Results are shown in table 2. No change in blood enzyme activity was observed following 6-azauracil administration. The dogs also showed no evidence of toxicity despite the high doses of 6-azauracil employed.

TABLE 2.—*Gal-1-P uridyl transferase activity in dog blood before and after administration of 6-azauracil*

Dog	Stage	Blood transferase activity*
1	Before 6-AZ	0. 005
	After 6-AZ	0. 006
2	Before 6-AZ	0. 004
	After 6-AZ	0. 007

*Expressed as μM of C^{14}-Gal-1-P incorporated into nucleotide per ml. RBC per hour.

Studies in Man

Materials and Methods

Galactose tolerance was measured before and during the chronic oral administration of 6-azauracil in three trials on two patients. One patient had metastatic liver involvement from a malignant argentaffinoma. He had normal routine liver-function studies, however, except for an abnormally high serum globulin and an elevated alkaline phosphatase. The other patient, with carcinoma of the lung, had no hepatic disease, on the basis of physical examination and routine liver-function tests.

For determination of galactose tolerance, these patients were fasted overnight. On the morning of the test, they were given 500 mg. of galactose per kg. body weight, as a 20 percent solution, administered intravenously over a period of 10 minutes. Serial blood samples were collected during a 3-hour period.

6-Azauracil was administered orally throughout the study at a dosage of 15 mg. per kg. per day. Galactose tolerance tests were performed after the patients had received 6-azauracil for at least 1 week. Both patients demonstrated either clinical or electroencephalographic evidence of 6-azauracil neurotoxicity at the time of these galactose-tolerance tests (*13*). No clinical antitumor effects were observed by attending physicians during the course of drug administration to these patients (*6*).

Gal-1-P uridyl transferase activity was measured in 5 other patients receiving 6-azauracil treatment. The dosages employed in this therapy ranged from 9 to 36 mg. per kg. body weight per day. Blood specimens were obtained prior to and during the course of drug administration.

Results

No significant change in galactose tolerance during 6-azauracil therapy was demonstrated in comparison with the baseline tests on the same patients. Indeed, there is a suggestion from the data that, if anything, a more rapid fall in galactose concentrations occurred in the tests performed during 6-azauracil administration.

Both patients had normal galactose tolerance tests initially; *i.e.*, blood galactose concentrations less than 20 mg. per 100 ml. 75 minutes after

intravenous injection (*14*). Although both were suffering from advanced carcinoma their disease was relatively static at the time of the study, and their general nutritional and clinical status did not undergo any apparent change in the interval between tests, save for the development of neurotoxicity secondary to the 6-azauracil.

Values for blood Gal-1-P uridyl transferase activity are given in table 3. There was no significant change in the enzymatic activity during 6-azauracil therapy, except in one patient, T. M. In this patient, the activity fell from a pretreatment value of 0.012 μM incorporation per ml. red blood cell per hour to a value of 0.002 μM after 14 days of 6-azauracil therapy. However, the patient became progressively more ill between the first and second measurements, and was near death at the time of the second determination.

TABLE 3.—*Gal-1-P uridyl transferase activity in human blood before and after 6-azauracil administration*

Patient	Diagnosis	Stage	Blood transferase activity*
F.B.	Acute lymph. leukemia	Before 6-AZ	0. 017
		6-AZ for 27 days	0. 015
E.J.	Carcinoma of breast	Before 6-AZ	0. 012
		6-AZ for 37 days	0. 013
J.B.	Subacute myel. leukemia	Before 6-AZ	0. 006
		6-AZ for 26 days	0. 005
M.W.	Chronic lymph. leukemia	Before 6-AZ	0. 003
		6-AZ for 14 days	0. 006
T.M.	Acute lymph. leukemia	Before 6-AZ	0. 012
		6-AZ for 14 days	0. 002

*Expressed as μM of C^{14}-Gal-1-P incorporated into nucleotide per ml. RBC per hour.

Discussion

Since 6-azauracil has been shown by microbiological studies to be a potential inhibitor of uracil and uridine, it was felt worthwhile to investigate its possible effects on galactose metabolism. As shown in text-figure 1, uridine diphosphoglucose is an essential reactant in the incorporation of galactose-1-phosphate into a nucleotide form. An antagonism by 6-azauracil or its metabolic derivatives in this reaction seemed, therefore, a possibility. However, in studies performed in mice, dogs, and man, administration of 6-azauracil produced no evidence of interference with galactose metabolism. Galactose tolerance was unchanged after the administration of large doses of 6-azauracil to dogs and mice, and after doses sufficient to produce typical signs of 6-azauracil toxicity in man.

The activity of the enzyme Gal-1-P uridyl transferase, catalyzing Gal-1-P incorporation, was similarly unchanged by drug administration. It should be pointed out that the assay would also measure, and consequently would not distinguish, the incorporation of galactose phosphate

into an abnormal azauridine nucleotide, perhaps one nonfunctional beyond this stage. However, an effect on the transferase reaction seems to be fairly completely ruled out.

Abnormality of galactose metabolism was demonstrated in a group of untreated mice suffering with advanced leukemia L1210. These animals, 7 days after tumor inoculation, were in a near-terminal state and had evidence of large axillary tumor masses and marked leukemic infiltration of the liver. This same group, 90 minutes after the intravenous injection of galactose, had elevated blood galactose concentrations, significantly higher that those of similarly treated normal mice. In contrast, another group of tumor-bearing mice which had received 6-azauracil for 6 days demonstrated no abnormal elevation of blood galactose following injection of this material. This treated group of animals also showed significant inhibition of local tumor growth, and had livers less markedly involved with leukemic infiltration.

These results indicate that mice with advanced leukemia manifest, among other metabolic abnormalities, an impaired ability to metabolize galactose. This may be related to the marked replacement of hepatic parenchyma by leukemic infiltrate seen in these animals. By exerting an inhibitory effect on the leukemia, 6-azauracil tends to preserve liver function and normal galactose metabolism.

Abnormalities in carbohydrate metabolism have been reported as common in humans suffering from cancer. A diabetic type of glucose-tolerance curve has been noted to occur in a significant proportion of patients with malignancy (*15*). The occurrence of a related abnormality in carbohydrate metabolism in mice with advanced leukemia, and the prevention of this defect by an antimetabolite, is demonstrated in this study.

Although the livers of the tumor-bearing mice showed marked leukemic involvement, especially in the animals not treated with 6-azauracil, the activity of liver Gal-1-P uridyl transferase was as high in this group as in the control animals. It does not appear that the demonstrated impairment of galactose metabolism seen in advanced mouse leukemia is related to decreased activity of this one particular enzyme. The defect responsible for the altered metabolism, however, could be present in one or several of the other enzyme systems involved in carbohydrate metabolism.

In the studies with patients, no changes in galactose tolerance were observed. There was likewise no change in their nutritional status during this interval and, of course, no chance to compare galactose tolerance with that existing prior to onset of carcinoma. However, the tolerance curves in both cases were within normal range.

In the five patients in whom blood Gal-1-P uridyl transferase activity was measured, four showed unchanged activity after 6-azauracil therapy; the fifth showed a decrease. This latter patient's clinical condition had steadily deteriorated between first and second determinations, and she was near death at the time the postdrug sample was obtained. The lowered enzyme activity, therefore, may be only a reflection of a more

generalized derangement of host metabolism secondary to severe terminal cachexia.

The findings of unchanged blood transferase activity in the other four leukemic patients tested does not absolutely preclude the possibility of changes in the transferase activity in the liver parenchyma of these patients. The reservation must be made that substantial changes in hepatic enzyme activity may have occurred which were not reflected by the measurements of enzymatic activity in blood. However, the unchanged levels in liver transferase activity in the mice treated with 6-azauracil render this possibility less likely.

Summary

1) No evidence was found for abnormal galactose metabolism associated with the administration of large doses of 6-azauracil. Galactose metabolism was measured by galactose tolerance tests and by assay of Gal-1-P uridyl transferase activity in blood or liver of mice, dogs, and man.

2) In near-terminal leukemic mice, with evidence of marked leukemic infiltration of the liver, blood galactose concentrations 90 minutes after intravenous administration of galactose were significantly elevated over concentrations seen in a control group. In similar tumor-bearing mice treated with 6-azauracil this elevation was not seen. The livers of the 6-azauracil-treated animals also showed less leukemic infiltration.

3) The previously reported tumor-inhibitory effects of 6-azauracil on mouse leukemia L1210 were confirmed.

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Observations on Tryptophan Staining of the Pancreatic *Alpha* Cells [1]

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Employing a new technique for the histochemical demonstration of protein-bound tryptophan, Glenner and Lillie recently described selective staining of pancreatic *alpha* cells, as well as other tissue sites including pancreatic zymogen granules, gastric chief cells, and crystalline lens (*1*). In the latter sites, in each case, proteins are known to occur having a tryptophan content of over 2 to 3 percent. It seems likely that such a protein is also to be found in the pancreatic *alpha* cells. Glucagon has a tryptophan content of 5.2 percent (*2*), and while the site of origin of this hormone has not been proved, the recent investigations cited by Elrick *et al.* (*3*) have provided convincing evidence that glucagon arises from the *alpha* cells of the pancreas and possibly from more ubiquitous tissues such as connective and lymphatic tissues.

The question naturally arises whether the material being stained in the *alpha* cells actually represents glucagon. The present study was undertaken to determine whether experimental procedures that might be expected to alter the glucagon content of the pancreas would induce appropriate changes in the tryptophan staining of the pancreatic *alpha* cells.

Materials and Methods

Animals used were New Zealand white female rabbits, weighing between 2 and 4 kilos. They were sacrificed by air embolus and 5 mm. portions of pancreas, duodenum, and stomach were removed and immediately placed in neutral buffered 4 percent formaldehyde and fixed for 6 hours. Tissue blocks were carried through the usual dehydration and embedding sequences, sectioned at 5μ and mounted on glass slides.

Demonstration of protein-bound tryptophan.—The method used for the demonstration of tryptophan was the postcoupled benzylidene reaction (*1*). In this method sections are fixed in 10 percent calcium acetate

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formalin for 6 hours and carried through the usual dehydration and paraffin-embedding procedures and then treated in the following manner:

1) Section at 5μ and deparaffinize.
2) Place in saturated picric acid solution for 24 hours, wash for 25 minutes in running water (this step enhances the staining reaction, but may be omitted).
3) Take sections (through graded alcohol if step 2 is used) to absolute alcohol and air-dry for 30 seconds.
4) Treat 5 minutes at 25° C. in: *p*-dimethylaminobenzaldehyde, 1 gm. in 10 ml. HCl (sp. gr. 1.19) and 30 ml. glacial acetic acid.
5) Wash gently 3 times in glacial acetic acid 30 seconds, 1 minute, 1 minute.
6) Place in Coplin jar that contains 40 ml. glacial acetic acid to which 1 ml. of a fresh diazotate of 8-amino-1-naphthol-5-sulfonic acid (S acid) has been added,[5] for 5 minutes at 25° C.
7) Wash gently 3 times in glacial acetic acid 1 minute each.
8) Clear through graded glacial acetic acid/xylene sequence to xylene 2 times and mount in Permount.

Identification of alpha *and* beta *cells.*—Various methods were used for the demonstration of specific cell types in the islets; these included the Gomori trichrome (*4*), aldehyde-fuchsin (*5*), azure A-eosin B at *p*H 4.5 (*6*), and the rhodocyan (*7*) techniques. Identification of islet cells containing tryptophan was achieved by first staining with the rhodocyan or azure A-eosin B techniques. The islets were photographed, the stain was then removed, and the islets were restained by the specific tryptophan methods.

Sites containing tryptophan stain a deep blue. Verification of the localization of tryptophan was afforded by two other specific techniques; the rosindole (*8*) and the xanthydrol (*9*) reactions.

Glucagon models.—Five mg. crystalline glucagon [6] was suspended in 2 cc. distilled water producing a cloudy white solution. To this was added 2 cc. neutral buffered 4 percent formaldehyde. After standing for 3 hours, the solution was centrifuged, and the supernatant was removed and tested for tryptophan (*10*). The precipitate was washed thoroughly and tested in a similar manner. Model slides were made by the addition of glucagon to a 3 percent aqueous solution of gelatin subsequently fixed in neutral formaldehyde for 6 hours. The model sections were prepared and stained in a fashion identical to the tissue sections.

Animal experiments.—Alloxan diabetes was produced by the rapid intravenous administration of alloxan monohydrate in a dose of 170 to 190 mg. per kilo. Insulin shock was produced by the subcutaneous injection of glucagon-free insulin in doses of 1.8 to 2.0 units per kilo. Blood sugars were determined by a modification of the Folin micromethod (*11*), and read on a Klett-Summerson photoelectric colorimeter. Sections were coded independently by the authors and examined without prior knowledge of the experiment performed on the individual animals.

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[5] This diazotate is made by adding 240 mg. of S acid to 3 ml. of *N*/1 HCl and 6 ml. of distilled water, cooling to 4° C., adding 1 ml. of *N*/1 $NaNO_2$, and stirring at 4° C. for 15 minutes.

[6] Obtained through the courtesy of Dr. W. R. Kirtley, Eli Lilly and Company Research Laboratories, Indianapolis, Ind.

Results

Localization of Protein-Bound Tryptophan

When tryptophan staining was followed by specific *beta*-cell staining with aldehyde-fuchsin, it was seen that the former method identified cells other than *beta* cells. These cells were shown to be exclusively *alpha* cells by prestaining with specific *alpha*-cell techniques. Zymogen granules of the acinar cells, duct content, and mucous cells were the only other elements of the pancreas prominently stained. In the gastrointestinal tract no staining of the enterochromaffin cells was noted (*1*, *5*).

Glucagon Precipitation

Glucagon was immediately precipitated by formaldehyde and, after 3 hours' exposure to the fixative, no tryptophan was detected in the supernatant although the precipitate was strongly positive. Microscopic examination of sections of the glucagon-containing gelatin models revealed numerous hexagonal crystals throughout. Following histochemical reactions for tryptophan, an intense blue coloration of the crystal was noted without diffusion of color into the surrounding gelatin or loss of hexagonal crystal structure (fig. 1).

Control Animals

Eight nonfasting rabbits were sacrificed; their tissues fixed and stained as above. The cytoplasm of the *alpha* cells stained a diffuse or finely-granular blue color, the cells being sharply delineated from surrounding structures (fig. 2). There was little variation from one animal to another. No staining of the *beta* cells was observed.

Alloxan Hyperglycemia

Seven rabbits were made diabetic by the intravenous administration of alloxan. Two animals were sacrificed after 2 days, 3 after 4 days, and 2 after 5 days. Their blood sugars just prior to death were 412, 287, 428, 482, 464, 500 and 562 mg. percent, respectively. Six of the 7 animals exhibited a definite increase in the intensity of staining of the *alpha* cells (fig. 3). This was not to be confused with a general shrinkage of the islet due to destruction of the *beta* cells, which might in general render *alpha*-cell staining more apparent than in the normal islet. In the seventh case the stain was described as not unlike the control group.

Alloxan Hypoglycemia

A second group of 6 animals was similarly given alloxan and was sacrificed during the hypoglycemic phase of the classic triphasic blood-sugar response to alloxan. Thus 6 to 9 hours after injection, blood sugars were determined and the animals sacrificed. The blood-sugar values in 5 animals were 36, 74, 36, 28 and 39 mg. percent. The sixth animal died

in opisthotonus before a blood sugar could be drawn. In all but one of these animals the intensity of *alpha*-cell staining was definitely decreased when compared with controls (fig. 4). When compared to the hyperglycemic alloxanized animals, the difference becomes all the more striking. At first glance, it was difficult to identify islet staining at all. On closer inspection, individual cells or groups of faintly-stained *alpha* cells could be identified. In the sixth animal, the stain was thought to be similar to the control group.

Insulin Shock

Hypoglycemic shock was produced in 5 rabbits by the subcutaneous administration of insulin. Blood sugars obtained just prior to death were 55, 40, 35, 30 and 20 mg. percent. All animals convulsed. In 2 other rabbits massive doses of insulin were given (80 units per kilo) and the animals sacrificed 2½ hours after injection. Six of the 7 animals showed staining similar to that of the control group. The seventh appeared somewhat increased.

Glucose Loading

Three rabbits were given a clysis of 1 gm. of glucose every 2 hours, for 6 hours, and sacrificed ½ hour after the last injection. Three other animals were given 2 gm. of glucose IV and sacrificed after 1 hour. Tryptophan staining of the *alpha* cells in the first group of animals was similar to the controls. In the second group, 2 of the 3 animals exhibited a slight decrease in the intensity of staining, but this was not thought to be significant.

Discussion

The histochemical demonstration of protein-bound tryptophan in *alpha* cells as well as in crystalline glucagon, fixed, precipitated, and stained in an identical fashion, suggests that variations in staining intensity of *alpha* cells may reflect changes in glucagon content of the pancreas.

Assaying for glucagon, Fodden and Read (*12*) have shown that extracts from the alloxan diabetic rabbit pancreas produce a more sustained hyperglycemia in the intact rabbit than does normal pancreas pretreated with cysteine to inactivate insulin. The results of our experiments reveal that in alloxan diabetic rabbits, the protein-bound tryptophan staining of the pancreatic *alpha* cells is markedly increased, while a commensurate increase in staining in other pancreatic elements shown to contain tryptophan does not occur.

The cross-circulation experiments of Foa (*13*) have shown that when a donor dog is made hypoglycemic with insulin, a hyperglycemic factor is released in the pancreatic vein blood which raises the blood sugar of the recipient dog. Since it is not known whether insulin *per se* or the resulting hypoglycemia was responsible for this release of "glucagon" from the pancreas, our experiments utilized two means of producing

both hyperglycemia and hypoglycemia. As previously noted, hyperglycemia in the absence of endogenous insulin (alloxan diabetes) caused a marked increase in tryptophan staining of *alpha* cells. However, acute hyperglycemia produced in the presence of intact *beta* cells by glucose loading, afforded no demonstrable change in *alpha*-cell staining. Hypoglycemia produced during the initial phase of alloxan treatment caused a significant decrease in this staining, while hypoglycemia induced by exogenous insulin failed to alter the histological picture substantially.

These latter findings are difficult to interpret, for if the decreased tryptophan staining during the hypoglycemic phase of alloxan treatment were due to hypoglycemia alone, a similar histological picture should be produced by insulin shock, as the blood sugars obtained were comparable in the two groups. Such was not the case. While it would appear that the insulin causes a release of a hyperglycemic factor from the pancreas, it cannot be concluded that the glucagon content of the pancreas is necessarily altered. In view of their proximity to necrosing *beta* cells, the *alpha* cells may have suffered transient injury during alloxanization. The increase in *alpha*-cell staining noted in the diabetic animal was not reproduced by acute glucose loading in the intact animal, suggesting that a longer period of hyperglycemia may be necessary to produce significant changes in the tryptophan content of the *alpha* cells.

While these preliminary studies suggest a relationship between histochemically demonstrable tryptophan in the *alpha* cells and glucagon content of the pancreas, it is conceivable that tryptophan-containing proteins other than glucagon contribute significantly to this staining quality of the *alpha* cells. Proof of the correlation between *alpha*-cell tryptophan and glucagon content await the application of a sensitive assay method for glucagon and a specific staining reaction such as antibody localization.

Summary

1) Protein-bound tryptophan can be demonstrated histochemically both in rabbit pancreatic *alpha* cells and in gelatin model slides of cystalline glucagon.

2) Protein-bound tryptophan staining of pancreatic *alpha* cells is decreased during the hypoglycemic phase and increased in the diabetic phase after alloxanization.

3) Insulin shock and acute glucose-loading failed to alter this staining quality significantly.

4) The possibility is discussed that the tryptophan containing protein demonstrable in the pancreatic *alpha* cells may be glucagon.

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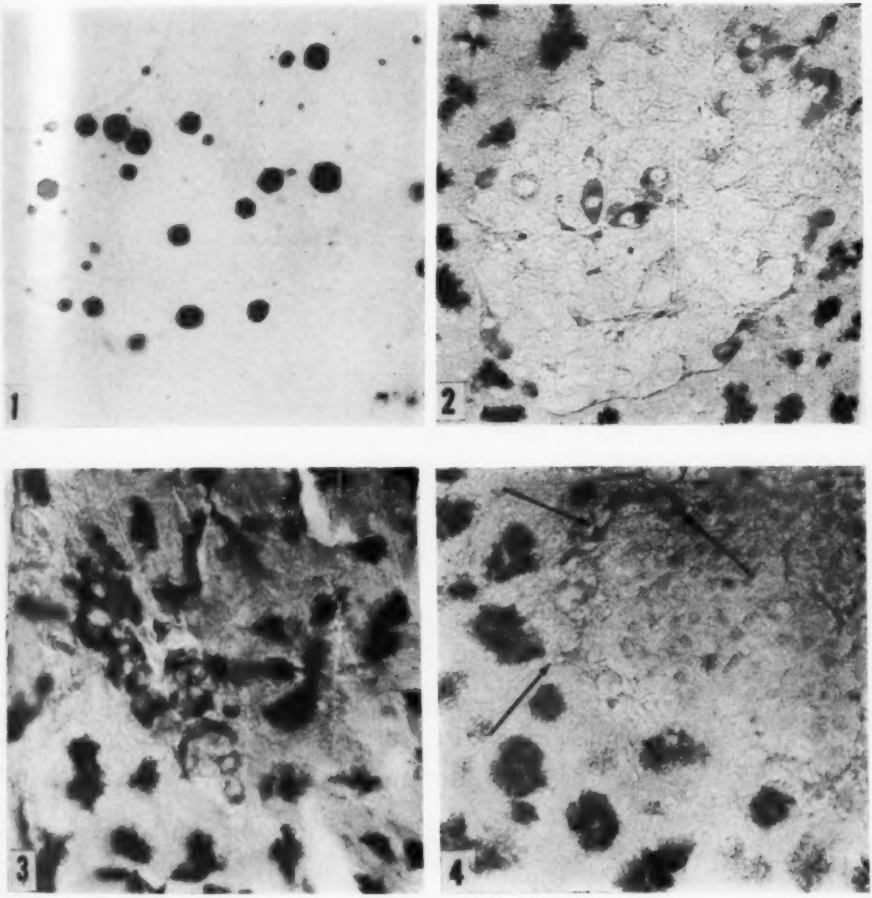
PLATE 8

FIGURE 1.—Crystals of formaldehyde-glucagon precipitate. *Note* lack of diffusion. Postcoupled benzylidene method (*red filter*). × 1230

FIGURE 2.—Normal pancreas. Tryptophan staining of *alpha* cells of islet. Zymogen granules of acinar cells are also stained. Postcoupled benzylidene method (*red filter*). × 860

FIGURE 3.—Alloxan hyperglycemia. Intense tryptophan staining of *alpha* cells with insular collapse following *beta*-cell destruction. Postcoupled benzylidene method (*red filter*). × 860

FIGURE 4.—Alloxan hypoglycemia. Marked diminution of tryptophan staining of *alpha* cells (*arrows*). Postcoupled benzylidene method (*red filter*). × 860



Levine and Glenner

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A Method for the Detection of *N*-Formiminoglutamic Acid in Urine [1]

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Broquist (*1*) has reported evidence for the occurrence of *N*-formiminoglutamic acid (FIGlu) in the urine of children with acute leukemia who were receiving folic acid antagonist therapy. Other reports have dealt with the excretion of this formimino derivative by the folic acid-deficient rat and by one subject with macrocytic anemia of pregnancy (*2–4*). In each instance, evidence for the occurrence of FIGlu was based in part on its conversion to glutamic acid by heat or alkali. These methods lack sensitivity and cannot be applied directly to human urine.

Since the occurrence of FIGlu in the urine of the rat has been shown to be related to folic acid deficiency (*3*), a sensitive and reasonably specific method for its determination might be useful in studies concerned with the action of antifolic acid compounds in man. We shall describe a procedure for the detection of FIGlu, which is based on the initial observations of Miller and Waelsch (*5*) and Slavík and Matoulková (*6*) that in crude liver extracts FIGlu acts as an effective formylating agent in the over-all conversion of tetrahydrofolic acid (THFA) to N^{10}-formyl THFA.

The procedure consists of (*a*) the enzymatic reduction of folic acid (FA) to THFA (*7*), (*b*) the enzymatic formylation of THFA (*5, 6, 8–10*) by FIGlu, (*c*) the nonenzymatic conversion of the product N^{10}-formyl THFA to N^5-formyl THFA (citrovorum factor, CF) (*11*), and (*d*) the microbiological assay of the N^5-formyl THFA (*11*). This procedure is applied directly to untreated urine.

Materials and Methods

CaCF, which had previously been isolated from liver, was used as a reference standard (*12*). FIGlu, as the barium salt ($C_6H_9N_2O_4Ba\frac{1}{2}\cdot 3H_2O$), was isolated from the urine of folic acid-deficient rats (*3*). Urocanic acid was synthesized and supplied by Dr. Howard Bond. The preparation of dialyzed chicken-liver extract was previously described (*7*).

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[3] National Cancer Institute.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare

A stock solution containing each of the following ingredients per ml. was prepared and stored in the frozen state: 0.55 μM of folic acid; 200 μM of KH_2PO_4 adjusted to *p*H 6.0 with KOH; 200 μM of citric acid adjusted to *p*H 6.0 with KOH; 200 μM of $MgSO_4 \cdot 7\ H_2O$ and 0.12 μM of triphosphopyridine nucleotide. To 1 ml. of the thawed solution, 0.5 ml. dialyzed chicken-liver extract was added and the mixture incubated for 2 hours at 37° C. By this time approximately 90 percent of the FA had been reduced to the tetrahydro level. Urine (usually 0.1 or 0.2 ml.) or 0.4 ml. of a standard solution of FIGlu containing 0.034 μM per ml. was added and the volume made to 2.3 ml. with water. In addition, digests made to the appropriate volume with water were included as reaction blanks. The incubation was continued for 2 additional hours. Ascorbic acid solution, 0.5 ml. (568 μM per ml. adjusted to *p*H 6.0), was then added to each tube and the samples were autoclaved at 10 pounds pressure for 30 minutes. Each sample was diluted to 100 ml. and this solution was diluted further for CF assay (*11*).

CF in each digest was determined and the values were corrected for the amount of CF found in the reagent blank. The FIGlu equivalents were calculated from the corrected CF values. A standard amount of FIGlu was included in each group of determinations as a check on the procedure.

The urine analyzed was obtained from a 48-year-old white male (O.R.) who had numerous basal-cell carcinomas involving the skin of the scalp, face, and back. He was ambulatory, in good nutritional status, and had no evidence of hepatic or renal disease. During the 50-day period of observation, he ate the regular hospital diet and was given no vitamins or other supplements. The patient's treatment was part of a cooperative study of one of us (P.T.C.) and Dr. E. J. Van Scott on the effect of the folic acid antagonists in cutaneous malignancy. He was treated with amethopterin (4-amino-N^{10}-methylpteroylglutamic acid), 7.5 mg., administered as a single daily oral dose for 15 days. Treatment was stopped because of the appearance of drug toxicity as manifested by oral ulcerations and depression of the formed elements of the peripheral blood. After a recovery period of 14 days, during which there was complete subsidence of the toxic signs, a second course of treatment with amethopterin was begun. This time the drug was given as a single daily subcutaneous injection of 7.5 mg. for 16 days. Once again cessation of therapy was dictated by the appearance of drug toxicity, which rapidly subsided, as before, after treatment was stopped. Two days after the last dose of amethopterin, he was given 2 oral doses of 0.5 gm. of histidine 12 hours apart. Urine samples were refrigerated without perservative immediately after voiding. Aliquots of 24-hour collections were frozen for subsequent analyses.

Results

Under the experimental conditions described, the active 1 carbon unit of FIGlu is quantitatively transferred to THFA (table 1). The experimental blank values, found in the absence of added FIGlu, are indicated

TABLE 1.—*Barium formiminoglutamate and citrovorum factor formed**†‡

Trial	(*a*) BaFIGlu taken for analysis	(*b*) CF found blank	(*a*+*b*) Theory	CF found
(1)	3. 23 (2)	2. 95 (2)	6. 18	6. 58
	6. 45 (2)	"	9. 40	8. 68
	12. 90 (2)	"	15. 85	14. 81
(2)	6. 45 (4)	2. 96 (2)	9. 41	9. 40
	12. 90 (4)	"	15. 86	15. 95
(3)	12. 90 (4)	3. 80 (4)	16. 70	17. 60
(4)	12. 90 (4)	2. 89 (4)	15. 79	17. 80

*All values in mμM.
†The values in parentheses indicate the numbers of replicates taken.
‡Average values of the replicates are shown in the table.

in column b. The variations between the calculated theoretical values and the values found are in part due to the normal variations expected of microbiological assays.

The results of the application of the procedure to several aliquots of one sample of urine from a patient receiving amethopterin are shown in table 2A. The values obtained from the analysis of the 5 aliquots of urine are in reasonable agreement. Further, it indicates that up to 0.5 ml. of urine may be analyzed without inhibiting the system. This specimen was analyzed for FIGlu at 5-day intervals. The values found are shown in table 2B and indicate that the results are reasonably reproducible.

The daily urinary-excretion pattern of FIGlu in the urine of a patient (O.R.) is shown in table 3. The subject excreted significant amounts of FIGlu 48 hours after therapy was initiated, and continued to excrete

TABLE 2: A.—*Application of method to urine aliquots**†

Ml. urine.....	0. 1	0. 2	0. 3	0. 4	0. 5
μM◇FIGlu in aliquot...	0. 0054	0. 0104	0. 0169	0. 0240	0. 0279
μM◇FIGlu per ml.....	0. 054	0. 052	0. 056	0. 060	0. 056
μM◇FIGlu excreted per day.....	47. 0 (2)	45. 3 (2)	48. 7 (2)	52. 2 (2)	48. 7 (2)

B.—*Reproducibility*

Trial number	1	2	3	4
μM◇FIGlu per day.....	53. 9 (1)	47. 3 (10)	47. 3 (2)	48. 7 (10)
Range of individual values, μM....		41. 2–51. 5	44. 6–50. 0	41. 3–54. 5

*Urine from patient, O.R. (day 17). Volume, 870 ml. excreted in 24 hours.
†Average values shown. Number of replicates indicated in parentheses.

substantial amounts of what corresponded in activity to FIGlu for at least 4 days after therapy was stopped (days 16 to 19). Two weeks after cessation of treatment the urinary excretion had returned to the original level. However, within 48 hours after the second course of therapy, the FIGlu excretion was increased. After the ingestion of 1 gm. of histidine, the patient excreted 188 μM equivalents of FIGlu. The increase in excretion of FIGlu after ingestion of histidine is consistent with previous observations made with the folic acid-deficient rat (*13*) and with one case of megaloblastic anemia of pregnancy (*4*). However, after the ingestion of 2 gm. of histidine by a normal adult male (44 years, M.S.) no FIGlu could be detected in the urine voided during the following 24 hours.

TABLE 3.—*Excretion of formiminoglutamic acid in urine of a subject receiving amethopterin*

Patient O. R.

Day	Treatment	μM FIGlu excreted per day	Day	Treatment	μM FIGlu excreted per day
0		9.8	29		6.4
1		6.1	30		11.5
2		17.5	31		19.6
3		14.5	32		31.1
4		14.9	33		27.0
5		19.9	34		27.4
6	7.5 mg. amethopterin per day given by mouth.	20.9	35	7.5 mg. amethopterin per day given subcutaneously.	31.1
7		25.0	36		34.7
8		21.9	37		38.2
9		18.6	38		35.5
10		28.4	39		25.7
11		38.8	40		44.3
12		36.9	41		39.5
13		35.2	42		45.7
14		34.3	43		88.8
15	None	34.3	44		46.0
16	"	34.5	45	None	70.9
17	"	47.7	46	1 gm. histidine	188.0
18	"	40.9	47	None—no urine collection.	—
	No treatment between days 15 and 29.		48	" " "	—
			49	None	44.7

The pattern of FIGlu excretion in the urine of the folic acid-deficient rat has previously been established (*3*). In the earlier studies, glutamic acid arising from the heat degradation of the formimino compound was used as an index of the concentration of FIGlu. The pattern of FIGlu excretion determined by the present method is shown in table 4. The diets and type of rats used were those described previously (*3*). Insignificant amounts of activity were excreted by the control animals. A progressive increase in activity, with time, is demonstrated by the two deficient animals. A sharp decrease occurred within 48 hours after the administration of folic acid. These results are in accordance with the previous observations in which the less sensitive technique based on the release of glutamic acid from FIGlu was employed.

TABLE 4.—*Occurrence of FIGlu activity in the urine of folic acid-deficient rats*

Rat	Diet	Time from start of experiment (*days*)								
		1*	3	6	10	13	17	20†	21†	24†
H-17‡	FA deficient	0. 26	0. 28	0. 91	7. 26	17. 50	30. 90	—	0. 41	0. 51
H-18	FA deficient	0. 16	0. 18	0. 56	1. 68	3. 42	13. 00	—	1. 42	0. 51
H-19§	Control	0. 08	0. 23	0. 15	0. 28	0. 23	0. 69	—	0. 25	0. 46
H-20	Control	0. 13	0. 23	0. 28	0. 46	0. 46	0. 46	—	0. 25	0. 25

*Values in μM FIGlu excreted per day.
†20 μg. of folic acid per gm. of diet to H-17 and H-18.
‡Average food intake for rats H-17 and H-18, 5.9 gm. per day.
§Average food intake for rats H-19 and H-20, 7.2 gm. per day.

Discussion

The data found in the study of the urine of a single patient are cited to indicate the type of information that may be obtained by the application of this method. The procedure should be helpful in chemotherapeutic studies involving antifolic acid compounds since FIGlu excretion appears to be related to FA deficiency.

Urine specimens from 4 other adult subjects receiving antagonist therapy have been analyzed for FIGlu activity. In one of these, an excretion pattern similar to that of the male (O.R.) reported in this study was noted. In another, no FIGlu could be detected until the subject was given a test dose of histidine. In the other two cases (subjects fed the normal hospital diet), no FIGlu could be detected.

Some consideration should be given to the limitations of the procedure when applied to human urine. Excretion values of less than 10 μM equivalents of FIGlu per day are of doubtful significance. The urine of patient O.R. (table 3) contained a substance equivalent to about 10 μM per day which, though not FIGlu, appeared as such in the analytical procedure. For example, the urine collection of day 17, after exposure to alkali,[5] which completely hydrolyzed FIGlu, gave values corresponding to 7.1 μM per day. Analysis of the untreated specimen indicated the presence of 47.6 μM as the day's output. Of the urine samples considered active (increase in FIGlu activity over the basal level) each sample tested showed a loss in activity of 75 to 90 percent after it was autoclaved for 10 minutes at 10 pounds pressure. This parallels the behavior of FIGlu in solution at *p*H 6.0 to 8.0. In practice, we assess the activity of urine specimens both before and after autoclaving and the presence of FIGlu is assumed only for those samples which lose the bulk of their activity after the heat treatment. For example, urocanic acid under our test conditions is fully as active as FIGlu (*5*) but loses no activity after autoclaving.

An unequivocal demonstration that the heat-labile material in human urine excreted during antifolic acid therapy is FIGlu must await its

[5] Five ml. of urine +1 ml. of 28 percent NH_4OH was stored for 1 hour at 23° C., concentrated to 0.5 ml. *in vacuo*, and the volume restored to 5.0 ml.

isolation in significant amounts and characterization. This has been done for the folic acid-deficient rat (*3*, *13*, *14*). The properties of the material as it exists in human urine are similar to those of FIGlu as it occurs in rat urine. Support for the view that the substance measured in human urine is FIGlu is based on the following indirect evidence. The active material in urine: 1) In the presence of metabolically generated THFA and in the absence of adenosinetriphosphate gives rise to N^{10}-formyl THFA; 2) is retained by Dowex 50-H^+; 3) is inactivated on autoclaving at *p*H 6.0 to 8.0; 4) is degraded by alkali; 5) occurs in increased amounts during folic acid deficiency of the host; and 6) is excreted in significantly increased amounts after the ingestion of histidine by the folic acid-deficient host. These properties exclude the following from consideration as the active factor(s) in urine: formic acid, methionine, histidine, urocanic acid, glycine, serine, and *N*-formyl glutamic acid. Miller and Waelsch (*5*) using a similar liver extract have shown that formic acid, formylisoglutamine, formylglutamine, and formylglutamic acid are inactive. The possibility has not been excluded that precursors or metabolically active derivatives of purines and thymine exist, which possess many of these properties and could contribute to the apparent FIGlu activity of urine. However, after a test dose of histidine, the only apparent product responsible for the increase in urine activity is FIGlu (*13*).

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Cancer Mortality Among Native White, Foreign-Born White, and Nonwhite Male Residents of Ohio: Cancer of the Lung, Larynx, Bladder, and Central Nervous System [1, 2]

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A previous study (*1*) showed an excess of lung-cancer mortality among foreign-born white compared with native white male residents of metropolitan areas of Ohio. This observation was derived from the consideration that the foreign born constitute a significant proportion of the adult population in urban areas and it was desired to determine whether this population subgroup influenced cancer mortality among adult males of these areas. It was recognized that the foreign born have tended to live and work together in the same sections of metropolitan areas and any association of abnormal cancer mortality with this population group would furnish the basis for more refined epidemiological studies. A more thorough exploration of the foreign-born mortality pattern seemed warranted, however, before undertaking such studies. The present study comparing cancer mortality among foreign-born white, native white, and nonwhite male residents of Ohio was made for this purpose. Available data are shown for four cancer sites—lung, larynx, bladder, and central nervous system.

Relatively few studies of cancer mortality among the foreign born are available. A study in Massachusetts, conducted by Lombard and Doering, compared cancer mortality for certain sites among the foreign born, native born of native parents, and native born of foreign parents for the period 1920–24 (*2*). Data published from this study, however, did not include the cancer sites considered in the present paper. Data for New Zealand, published by Eastcott in 1954, compared cancer mortality among the native-born population and immigrant groups born in Great Britain. He found an excess in mortality among the immigrants for cancer of the lung and bronchus but not for nine other sites considered (*3*). His data did not include cancer of the bladder or tumors of the central

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nervous system. Steiner made a study of necropsies in California and observed racial differences in mortality for certain cancer sites. He also made a comprehensive review in which he drew attention to specific deficiencies in the ethnic and geographic data (*4*). A recent study has shown an excess of lung-cancer mortality among Mexican women in California (*5*).

Some statistics on mortality among residents of various countries are available in the vital statistics reports of individual countries and in publications of the World Health Organization (*6*, *7*). Such data serve as a limited basis of reference for observations on foreign-born residents of the United States. The National Office of Vital Statistics of the United States has also published some statistics on mortality in broad-cause groups, including total cancer, among foreign-born residents of the United States.

Available data indicate that mortality patterns by nationality exist, involving differences among countries and within individual countries.

Method of Study

The present study is devoted to mortality due to cancer of selected sites among native white, foreign-born white, and nonwhite males 25 to 64 years inclusive. The findings are limited to Ohio residents and are shown by age, urban-rural residence, selected urban counties of residence, selected countries of birth, and duration of residence.

Mortality Data

The study includes deaths due to cancer of the lung, larynx, bladder, and tumors of the central nervous system. The years covered by the data and the cause of death codes used for each cancer site studied are as follows:

Cancer site	*Death data for years*	*Cause of death code (International statistical classification of diseases, injuries, and causes of death)*	
		Fifth revision 1944–48	*Sixth revision 1949–51*
Lung (includes trachea and bronchus)	1947–51	47B, 47C, 47D	162, 163
Larynx	1947–51	47A	161
Bladder	1947–51	52B	181
Tumors of the central nervous system	1944–52	54A, 54B, 56D, 57D	193, 223, 237

Data on characteristics and place of residence of the persons who died were taken from death certificates. All deaths of Ohio residents who died in the State were included, but nonresident deaths occurring within the State were excluded.

Data on cancer of the lung, larynx, and bladder include all deaths reported on death certificates as due to these causes. Data on tumors of the central nervous system are limited to deaths for which this cause was reported on the death certificate and confirmed by available results

of histological examinations following autopsy or surgery. Although question has been raised about the accuracy of cause of death as recorded on death certificates, note has been made of the fact that there is a close similarity in cancer-mortality data from death certificates and confirmed sources such as autopsy or clinical records (*1*, *8*).

Data on duration of residence of persons who died of tumors of the central nervous system were derived from a special follow-up of these individuals, which included checking city directories and employment records, and queries of relatives and others knowing the deceased.

Population Data

Population data used as a basis for computing death rates for Ohio, as well as the United States, were taken from the United States decennial censuses (*9*, *10*).

Estimates were necessary to obtain 1950 data for counties in Ohio on the age distribution of the total native and foreign-born white males and the age and sex distribution of males of specific nativity groups. The estimates were made on the basis of 1950 Census data on the characteristics of the foreign born of Standard Metropolitan Areas, as defined by the 1950 Census. The procedures used were as follows:

The age distribution of the native and foreign-born white males in each of the metropolitan counties of the State was estimated on the basis of the age distribution of the native and foreign-born white males in the Standard Metropolitan Area in which the county was located.

Available statistics on the age and sex distribution of eight individual nativity groups in the Cleveland Standard Metropolitan Area were used to estimate the number of males of selected ages in the corresponding nativity group in Cuyahoga County. The steps were as follows:

1) Determining the percentages of males in specific age groups among the total population of specified nativity groups in the Cleveland Standard Metropolitan Area.
2) Applying the resulting percentages to the total population of the appropriate nativity group in Cuyahoga County.

The procedure outlined was used for the Cuyahoga County estimates for persons born in England and Wales, Ireland, Germany, Poland, Czechoslovakia, Austria, U.S.S.R., and Italy. Estimates of males in specific age groups for selected other European countries of birth were made by the same procedure on the basis of the age and sex distribution of the population of a neighboring European country for which an age and sex distribution was available in the Cleveland Standard Metropolitan Area statistics.

Definitions

The following definitions and methods of reporting information were used as a basis for data in the study:

1) Nonwhites include Negroes, Indians, Japanese, Chinese, and other nonwhite races.

2) Native-born persons include persons born in the United States or any of its territories or possessions.
3) Country-of-birth data for the deaths are based on the report of the person supplying information for the death certificate, and nativity data for the population statistics are based on the answer of the respondent to a question on the state or foreign country of his birth. The nativity data are classified on the basis of international boundaries in 1950. It is probable, however, that some of the reported information is in terms of boundaries of countries at a previous time. This point is of special importance in interpretation of the nativity data for central European countries because of the numerous boundary changes there since some of the persons studied were born.
4) Age groupings are based on the age of the person at his last birthday. A note on population data in the 1950 Census indicates that ". . . there appears to be a deficit of persons in the age range 55 to 64 years, which, however, is more than offset by an excess over the number expected in the age group 65 years old and over." (*11*). In discussing life table data for 1940, Greville noted particularly among Negroes that there appeared to be an overstatement of age groups just beyond 65 at the expense of those just under that age, both in census and mortality statistics. Greville suggested that the provision of Social Security benefits for persons over 65 probably accounted for the misstatement of age (*12*).
5) The residence used for both mortality and population data is the usual place of residence.
6) The distinction between urban and rural residence is based on the 1950 Census definition. That is, "The urban population comprises all persons living in (a) places of 2,500 inhabitants or more incorporated as cities, boroughs, and villages, (b) incorporated towns of 2,500 inhabitants or more . . ., (c) the densely settled urban fringe, including both incorporated and unincorporated areas, around cities of 50,000 or more, and (d) unincorporated places of 2,500 inhabitants or more outside any urban fringe. The remaining population is classified as rural." (*13*).
7) Metropolitan counties are defined as those with cities of 100,000 population or more in 1950.
8) A Standard Metropolitan Area is defined by the 1950 Census as ". . . a county or group of contiguous counties which contains at least one city of 50,000 inhabitants or more. In addition to the county, or counties, containing such a city, or cities, contiguous counties are included in a standard metropolitan area if according to certain criteria they

are essentially metropolitan in character and socially and economically integrated with the central city." (*14*).

Statistical Procedures

Statistical procedures used for analysis and presentation of the findings were as follows:

1) Average annual color, nativity, and age-specific death rates for each cancer site were computed for Ohio on the basis of the 1950 population of the appropriate population subgroup.
2) Age-adjusted death rates were computed by the direct method for each cancer site for selected population subgroups in the State of Ohio and in urban and rural areas of the State on the basis of the age distribution of the total population of continental United States in 1950.
3) Expected deaths due to cancer of the lung among the native white, foreign-born white, and nonwhite males were computed for each of the eight metropolitan counties by applying the observed age-specific lung cancer death rates of each color and nativity group in the combined eight metropolitan counties to the corresponding color, nativity, and age group in each county. Expected deaths among the native and foreign-born white males of each county were computed for the other three cancer sites by the same procedure.
4) Expected deaths were also computed by cancer site for the foreign-born white males of selected nativity groups in Cuyahoga County on the basis of the age-specific death rates of the total foreign-born white males in Cuyahoga County.
5) The number of expected deaths computed by the above procedures was compared with the number of observed deaths in the corresponding population subgroup for each cancer site.
6) Standardized mortality ratios were computed to assist in the comparison of observed and expected deaths in each country-of-birth group for cancer of the lung in Cuyahoga County. The ratios were computed by dividing observed deaths by expected deaths and multiplying the result by 100, *i.e.*:

$$\frac{\text{Observed deaths}}{\text{Expected deaths}} \times 100.$$

The resultant ratio expresses the average annual mortality rate of each nativity group, adjusted for age by the indirect method, as a percentage of the total eight-county rate for the foreign born.

Study Area

The State of Ohio, which is the area of study, had a total of almost 2 million males 25 to 64 years of age in 1950 (table 1). Eighty-five percent of these males were native white and the remainder were about equally divided between the foreign-born whites and the nonwhites. The proportion of males who were foreign-born white increased sharply with age, reaching approximately 20 percent in the 55-to-64 year age group.

TABLE 1.—*Color, nativity, and age distribution of males, 25 to 64 years of age, Ohio, 1950**

Color and nativity	Age group			
	25–64	25–44	45–54	55–64
	Number of persons			
Total	1, 977, 585	1, 153, 720	452, 130	371, 735
	Percent			
Total	100. 0	100. 0	100. 0	100. 0
White	93. 2	92. 8	92. 8	94. 9
Native	85. 2	89. 9	81. 6	75. 2
Foreign born	8. 0	2. 9	11. 2	19. 7
Nonwhite	6. 8	7. 2	7. 2	5. 1

**Source:* Based on data in U. S. Census of Population: 1950, Vol. II, Characteristics of the Population, Part 35, Ohio, p. 206, U. S. Department of Commerce, Bureau of the Census.

Approximately three fourths of the males 25 to 64 years of age resided in urban areas as defined by the 1950 Census. Data in table 2 show that the urban areas had somewhat higher proportions of foreign-born whites and nonwhites than the rural areas.

TABLE 2.—*Color and nativity distribution of males, 25 to 64 years of age, urban and rural areas of Ohio, 1950**

Color and nativity	Urban	Rural
	Number of persons	
Total	1, 430, 090	547, 495
	Percent	
Total	100. 0	100. 0
White	91. 3	98. 3
Native	81. 7	94. 6
Foreign born	9. 6	3. 7
Nonwhite	8. 7	1. 7

**Source:* Based on data in U. S. Census of Population: 1950, Vol. II, Characteristics of the Population, Part 35, Ohio, pp. 206-207, U. S. Department of Commerce, Bureau of the Census.

There are eight metropolitan counties, as previously defined, scattered over the State (map, text-fig. 1). The two most populous of these counties are Cuyahoga and Hamilton, which include the cities of Cleveland and Cincinnati and had total populations in excess of 500,000 in 1950. The

total number of males 25 to 64 years of age in the combined eight metropolitan counties was 1,157,329 in 1950.

The eight metropolitan counties followed the pattern noted for urban areas of having somewhat higher proportions of foreign-born whites and nonwhites among the males 25 to 64 years of age than other areas of the State. It is especially interesting that the foreign-born whites constituted approximately one fourth of the males in the 55-to-64 year age group in the combined eight metropolitan counties.

The color and nativity characteristics of the males 25 to 64 years of age also showed some variation among the individual metropolitan counties. For example, Cuyahoga and Mahoning Counties had relatively high proportions of foreign-born white males, offset by relatively low proportions of native white males. Franklin, Hamilton, and Montgomery

TEXT-FIGURE 1.—Eight metropolitan counties of Ohio, 1950.

Counties, on the other hand, had relatively few foreign-born white males and Stark County had a particularly low proportion of nonwhite males.

The distribution of the total foreign-born white population by selected countries of birth is available for each of the eight metropolitan counties in table 3. It can be seen that persons born in Italy, Germany, Poland, Hungary, and Czechoslovakia constituted the largest nativity groups, together accounting for approximately half of the foreign-born white population in the combined eight metropolitan counties. The nativity distribution of the foreign-born whites varied considerably among the counties. For example, persons born in Poland were relatively numerous in Lucas County and the Germans were relatively numerous in Hamilton and Montgomery Counties.

It is also of interest to know how long the foreign born have resided in this country. Data for the United States show that immigration was greatest in the period from 1880–1930. The decade with the largest number of immigrants was 1901–10. This, of course, suggests that most

TABLE 3.—*Country of birth distribution of the foreign-born white population for each of the eight metropolitan counties of Ohio, 1950**

Country of birth	Total 8 counties	County							
		Cuyahoga	Franklin	Hamilton	Lucas	Mahoning	Montgomery	Stark	Summit
		Number of persons							
All foreign countries	334,066	184,791	13,750	26,088	23,116	29,993	10,787	16,686	28,855
		Percent							
All foreign countries	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
England and Wales	5.8	4.8	9.4	4.8	5.6	7.4	7.1	6.8	8.6
Scotland	2.5	2.5	2.3	1.7	2.2	2.8	2.0	2.0	3.9
Ireland	2.9	3.3	3.2	4.4	2.5	2.0	2.9	0.9	1.5
Germany	11.0	8.1	16.9	31.2	17.6	3.0	26.4	9.1	6.3
Poland	10.2	12.4	3.0	3.4	22.3	6.5	5.2	4.1	5.2
Czechoslovakia	8.8	11.8	1.4	0.7	2.5	14.9	1.8	4.0	5.3
Austria	5.3	6.2	3.4	4.2	2.1	4.4	3.5	5.4	5.5
Hungary	9.9	11.0	5.0	7.1	7.9	7.2	11.6	6.3	13.9
Yugoslavia	6.2	6.9	2.5	1.9	0.7	7.5	3.4	4.5	12.0
U. S. S. R.	6.2	6.6	7.3	9.8	4.0	4.9	5.8	3.5	4.3
Lithuania	1.6	2.2	1.0	1.1	0.6	0.5	2.5	0.4	1.0
Rumania	2.2	1.6	1.3	2.8	0.8	3.6	1.0	8.3	2.1
Greece	2.2	1.2	3.6	2.3	2.8	3.3	3.6	6.3	3.1
Italy	12.2	10.8	19.1	10.3	3.6	23.6	6.1	19.1	13.1
Canada	4.5	4.4	6.1	3.9	9.3	1.5	6.4	2.9	4.1
All other countries	8.5	6.2	14.5	10.4	15.5	6.9	10.7	16.4	10.1

**Source:* Based on data in U. S. Census of Population: 1950, Vol. II, Characteristics of the Population, Part 35, Ohio, pp. 163–164, U. S. Department of Commerce, Bureau of the Census.

of the foreign-born residents of Ohio at the time of this study had been in this country for a considerable period of time (*15*). Census data on the number of foreign-born white male residents of Ohio in 10-year intervals from 1880 through 1950 show that the total reached a peak in 1920 and declined after that year.

Background Mortality Data

Data on over-all mortality in Ohio among the native white, foreign-born white, and nonwhite males 25 to 64 years of age are available by decade for the period from 1920–50 in table 4. The table shows that the age-adjusted mortality rate was somewhat higher among the foreign-born white males than among the native white males at each 10-year interval from 1920–50, and the difference decreased in the later decades. The rate among the nonwhite males was considerably higher than for either of the white male population groups in the same time periods.

TABLE 4.—*Annual age-adjusted death rate* per 1,000 native white, foreign-born white, and nonwhite males, 25 to 64 years of age, Ohio, 1920–50* †‡

Color and nativity	Year			
	1950	1940	1930	1920
Total	8. 25	9. 42	10. 69	10. 72
White	7. 85	8. 86	9. 94	10. 28
Native	7. 65	8. 62	9. 44	9. 60
Foreign born	7. 92	9. 93	11. 95	12. 72
Nonwhite	14. 05	19. 64	25. 29	21. 38

*Age adjusted each year on the basis of the total population of continental United States, 1950. Deaths are by place of occurrence for the years 1920–40 and by place of residence for 1950.

†During the period from 1920–50 there were minor changes in the procedure by which published data on mortality and population were grouped in the categories shown in the table. In the absence of mortality data for 1940, deaths for that year were calculated by taking one half of the published death totals for the period 1939–40.

‡ *Source* of basic mortality data:

(1) U. S. Department of Commerce, Bureau of Census: Mortality Statistics, 1920, 21st Annual Report, p. 202; Mortality Statistics, 1930, 31st Annual Report, p. 135; and Vital Statistics Supplement, 1939–40, Part III, Nativity and Mortality Data, p. 67.

(2) U. S. Department of Health, Education, and Welfare, Public Health Service, National Office of Vital Statistics: Vital Statistics of the United States, 1950, Vol. III, Mortality Data, p. 57.

The relative position of various male population subgroups in mortality rates for the United States is shown for 1950 in table 5. It is of interest to note that for the age span 25 to 64 years the age-adjusted mortality rate due to all causes of death was slightly higher among foreign-born white males than among native white males and was considerably higher among nonwhite males than in either of the white male population subgroups. Mortality due to malignant neoplasms was higher among both the foreign-born white and the nonwhite males than among the native white males in each age group from 25 to 64. It is of particular interest that mortality due to malignant neoplasms was somewhat higher among the foreign-born white males than among the nonwhite males in the 55-to-64 year age group. Such a change in the relative position of the foreign-born white and

TABLE 5.—*Death rate due to all causes and to malignant neoplasms per 1,000 native white, foreign-born white, and nonwhite male residents, 25 to 64 years of age, classified by age, United States, 1950**

Color and nativity	Age group				Age adjusted† 25–64
	25–64	25–44	45–54	55–64	
	All causes				
Total	8. 76	3. 22	10. 89	24. 53	8. 71
White	8. 24	2. 83	10. 06	23. 53	8. 11
Native	7. 40	2. 78	9. 86	22. 67	7. 89
Foreign born	14. 30	3. 13	10. 24	24. 94	8. 58
Nonwhite	13. 71	6. 76	18. 67	36. 88	14. 76
	Malignant neoplasms				
Total	1. 30	0. 31	1. 59	4. 23	1. 29
White	1. 29	0. 31	1. 54	4. 18	1. 27
Native	1. 09	0. 30	1. 46	3. 77	1. 17
Foreign born	2. 93	0. 40	1. 95	5. 39	1. 63
Nonwhite	1. 39	0. 36	2. 08	4. 84	1. 54

**Source:* Based on data in (1) Vital Statistics of the United States, 1950, Vol. III, Mortality Data, pp. 452, 454. U. S. Department of Health, Education, and Welfare, Public Health Service, National Office of Vital Statistics, and (2) U. S. Census of Population, 1950, Vol. II, Characteristics of the Population, Part I, United States Summary, p. 172, U. S. Department of Commerce, Bureau of the Census.

†Age adjusted on the basis of the total population of continental United States, 1950.

nonwhite population groups for deaths due to malignant neoplasms at ages 55 to 64 did not, however, appear among the females.

Data from the World Health Organization on cancer mortality in selected European countries are available in table 6. The statistics shown are limited to England and Wales, Ireland, the Federal Republic of Germany, and Italy, as well as the United States, for cancer of the lung, larynx, and bladder because of the limited availability of comparable data for other areas of the world. The mortality rates shown in table 6 do, however, suggest considerable variation in cancer mortality among countries. There are also differences in cancer mortality in various areas within other countries, as shown, for example, in studies conducted in Great Britain by Stocks and Fulton (*16, 17*).

Findings

The findings of the present study are summarized under five general headings: the State as a whole, urban-rural comparisons, county comparisons, country of birth comparisons, and duration of residence. Some of the comparisons for population subgroups and geographic areas are omitted for cancer of the larynx and bladder and tumors of the central nervous system because the limited number of deaths due to these causes made such detailed breakdowns seem inadvisable. The country-of-birth comparisons are limited to Cuyahoga County because of the lack of data on the age and sex distribution of specific nativity groups in other areas in which metropolitan counties are located.

In interpeting the mortality data, unadjusted for age, it should be noted that the foreign-born white males were relatively older than the native

white or the nonwhite males in the communities studied. As a result, the death rates by age group, especially in the larger span from 25 to 64 years, tend to be weighted more by the older persons in the group among the foreign-born white than among the native white or the nonwhite males. This is of importance because of the rapid rise with age of mortality due to cancer of the sites studied. The extent of the influence of the foreign-born age distribution, of course, varies with any differences in the age composition of the communities studied.

In an effort to overcome some of the difficulties in differences in age distribution of the native white, foreign-born white, and nonwhite population

TABLE 6.—*Death rate due to malignant neoplasms of selected sites per 100,000 males, 25 to 64 years of age, classified by age: England and Wales, Ireland, and United States, 1950; Italy, 1951; Federal Republic of Germany, 1952**

Country	Age group			
	25–64	25–44	45–54	55–64
	Cancer of the lung			
England and Wales	59. 14	10. 40	77. 37	180. 22
Ireland	18. 28	4. 29	26. 39	52. 25
Federal Republic of Germany	36. 23	3. 97	43. 92	113. 82
Italy	15. 76	3. 26	27. 94	45. 26
United States	25. 79	4. 01	35. 69	85. 78
	Cancer of the larynx			
England and Wales	2. 33	0. 22	2. 50	8. 43
Ireland	3. 48	1. 01	2. 93	12. 25
Federal Republic of Germany	2. 35	0. 30	2. 38	8. 00
Italy	4. 13	0. 69	6. 63	13. 46
United States	2. 27	0. 24	3. 16	7. 94
	Cancer of the bladder			
England and Wales	5. 42	0. 54	5. 58	19. 94
Ireland	2. 32	0. 00	3. 52	8. 16
Federal Republic of Germany	†	†	†	†
Italy	2. 87	0. 38	4. 10	10. 43
United States	4. 23	0. 41	4. 24	16. 99

**Source:* Based on mortality data supplied by the World Health Organization and population data in United Nations publications.

†Not available.

subgroups studied, death rates adjusted for age by the direct method or expected deaths were used for the age span 25 to 64. It should be noted that the resulting statistics are based on data grouped by ages 25 to 44, 45 to 54, and 55 to 64. To provide a supplementary exploration of the importance of using smaller age groups, age-adjusted death rates were also computed for cancer of the lung, larynx, and bladder in Ohio and urban and rural areas of the State on the basis of age groups 25 to 44, 45 to 49, 50 to 54, 55 to 59, and 60 to 64. The resulting age-adjusted rates based on the smaller age groups showed very minor changes in the foreign-born rates and negligible changes for the white and nonwhite population groups which did not alter the general conclusions.

State As A Whole

Color and Nativity

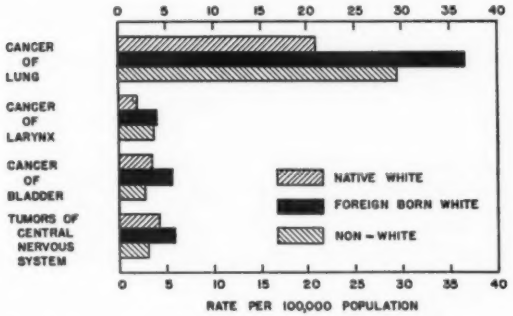
Mortality was higher among foreign-born white males than among native white males for each of the four cancer sites in the 25-to-64 year age range. The difference was relatively larger for cancer of the lung and larynx than for either of the other sites (text-fig. 2, tables 7 and 8).

The relative standing of mortality among the nonwhites compared with the 2 white population subgroups varied among the cancer sites. Age-adjusted mortality rates for ages 25 to 64 years for each of the population subgroups show that for cancer of the lung nonwhite mortality was about midway between mortality among the native white and the foreign-born white males. On the other hand, the nonwhites had an age-adjusted mortality rate similar to the foreign-born whites for cancer of the larynx, similar to the native whites for cancer of the bladder, and lower than either of the other two population subgroups for tumors of the central nervous system.

TABLE 7.—*Average annual death rate due to cancer of selected sites per 100,000 native white, foreign-born white, and nonwhite male residents of Ohio, 25 to 64 years of age, classified by age: Cancer of the lung, larynx, and bladder, 1947–51; tumors of the central nervous system, 1944–52*

Color and nativity	Age group				Age adjusted*
	25–64	25–44	45–54	55–64	25–64
	Cancer of the lung				
Total	24. 90	4. 04	35. 48	76. 78	23. 98
White	24. 72	3. 72	34. 90	76. 39	23. 59
Native	20. 01	3. 66	32. 59	64. 01	20. 85
Foreign born	75. 07	5. 29	51. 70	123. 77	36. 67
Nonwhite	27. 39	8. 22	42. 94	83. 92	29. 43
	Cancer of the larynx				
Total	2. 47	0. 43	2. 79	8. 39	2. 36
White	2. 39	0. 41	2. 48	8. 28	2. 26
Native	1. 86	0. 39	2. 17	6. 94	1. 94
Foreign born	7. 99	1. 18	4. 74	13. 42	4. 14
Nonwhite	3. 57	0. 73	6. 75	10. 49	3. 82
	Cancer of the bladder				
Total	3. 93	0. 35	4. 20	14. 74	3. 75
White	4. 00	0. 34	4. 20	14. 91	3. 78
Native	3. 42	0. 27	4. 12	14. 16	3. 58
Foreign born	10. 27	2. 35	4. 74	17. 80	5. 60
Nonwhite	2. 98	0. 48	4. 29	11. 54	3. 29
	Tumors of the central nervous system				
Total	4. 32	2. 74	5. 92	7. 26	4. 26
White	4. 41	2. 76	6. 14	7. 34	4. 34
Native	4. 17	2. 69	6. 02	7. 23	4. 25
Foreign born	6. 90	4. 90	7. 02	7. 76	5. 89
Nonwhite	3. 06	2. 42	3. 07	5. 83	3. 17

*Age adjusted on the basis of the total population of continental United States, 1950.


0 5 10 15 20 25 30 35 40
CANCER OF LUNG
CANCER OF LARYNX
CANCER OF BLADDER
TUMORS OF CENTRAL NERVOUS SYSTEM
NATIVE WHITE
FOREIGN BORN WHITE
NON-WHITE
0 5 10 15 20 25 30 35 40
RATE PER 100,000 POPULATION

TEXT-FIGURE 2.—Average annual age-adjusted death rate due to cancer of selected sites among native white, foreign-born white, and nonwhite male residents of Ohio, 25 to 64 years of age. (Age adjusted on the basis of the total population of continental U. S., 1950.)

Cancer of the lung, larynx, and bladder, 1947–51.
Tumors of the central nervous system, 1944–52.

TABLE 8.—*Number of deaths due to cancer of selected sites among native white, foreign-born white, and nonwhite male residents of Ohio, 25 to 64 years of age, classified by age: Cancer of the lung, larynx, and bladder, 1947–51; tumors of the central nervous system, 1944–52*

Color and nativity	Age group			
	25–64	25–44	45–54	55–64
	Cancer of the lung			
Total	2,462	233	802	1,427
White	2,278	199	732	1,347
Native	1,686	190	601	895
Foreign born	592	9	131	452
Nonwhite	184	34	70	80
	Cancer of the larynx			
Total	244	25	63	156
White	220	22	52	146
Native	157	20	40	97
Foreign born	63	2	12	49
Nonwhite	24	3	11	10
	Cancer of the bladder			
Total	389	20	95	274
White	369	18	88	263
Native	288	14	76	198
Foreign born	81	4	12	65
Nonwhite	20	2	7	11
	Tumors of the central nervous system			
Total	768	284	241	243
White	731	266	232	233
Native	633	251	200	182
Foreign born	98	15	32	51
Nonwhite	37	18	9	10

Age, Color, and Nativity

Mortality increased with age for each cancer site and population subgroup studied. The rise was, however, somewhat less marked for tumors of the central nervous system than for the other cancer sites (text-fig. 3, tables 7 and 8).

Color and nativity comparisons in mortality for age groups 25 to 44, 45 to 54, and 55 to 64 can be made for each cancer site in table 7. It is of interest that the excess in mortality among the foreign-born white males, compared with native white males noted above, was present in each of the age groups for each cancer site studied. The relative position of mortality rates among the nonwhite males compared with each of the white male population groups, however, showed some variation with age for each of the four cancer sites except tumors of the central nervous system.

In view of possible variations in cancer mortality which may occur in smaller age groups than shown in table 7, mortality rates for cancer of the lung were computed in 5-year age groups from ages 45 to 64, by color and nativity. The resulting lung-cancer death rates per 100,000 population were as follows for Ohio for the years 1947–51:

Color and nativity	*Age group*			
	45–49	*50–54*	*55–59*	*60–64*
Native white	22. 16	44. 28	55. 23	74. 83
Foreign-born white	25. 99	70. 72	109. 17	138. 17
Nonwhite	36. 10	51. 11	79. 16	90. 97

The data indicate that the general observations made for the larger age groups also hold in smaller age groups, and also show that the shift in relative position of the foreign-born white and nonwhite males appeared in the 50-to-54 year age group.

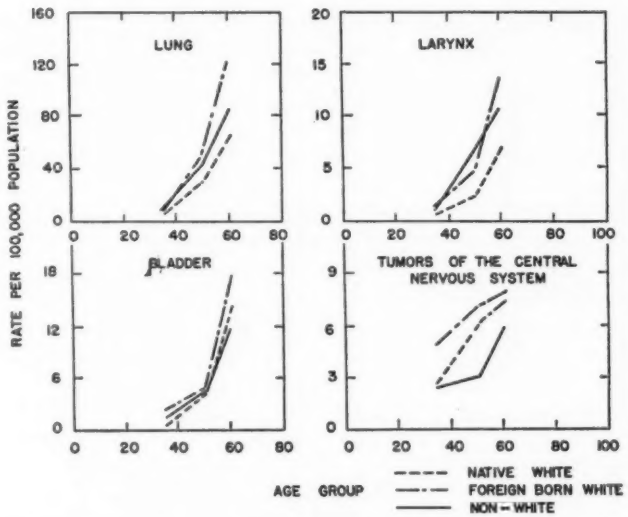
It is of interest to note in text-figure 3 that the excess in mortality among foreign-born white males compared with native white males was greatest in the 55-to-64 year age group for cancer of the lung but was concentrated primarily in the 25-to-44 year age group for tumors of the central nervous system.

Urban-Rural Comparisons

Mortality was generally higher in urban than in rural areas, as defined by the 1950 Census, for each of the cancer sites studied.

Urban-rural comparisons in age-adjusted mortality rates among native white males 25 to 64 years of age for each cancer site indicate that mortality was from 30 to over 100 percent higher in urban than in rural areas. The difference was greatest for cancer of the lung (table 9).

Urban-rural comparisons in mortality among the native white, foreign-born white, and nonwhite males 25 to 64 years of age are available for cancer of the lung in text-figure 4 and table 10. The data indicate that the age-adjusted, lung-cancer mortality rate among the native white as well as among the nonwhite group was from two to three times as great in urban as in rural areas. Foreign-born mortality for this cancer site,



TEXT-FIGURE 3.—Average annual death rate due to cancer of selected sites among native white, foreign-born white, and nonwhite male residents of Ohio, 25 to 64 years of age, classified by age.

Cancer of the lung, larynx, and bladder, 1947–51.
Tumors of the central nervous system, 1944–52.

TABLE 9.—*Average annual age-adjusted death rate* due to cancer of selected sites per 100,000 native white males, 25 to 64 years of age, residing in urban and rural areas of Ohio: Cancer of the lung, larynx, and bladder, 1947–51; tumors of the central nervous system, 1944–52*

Cancer site	Death rate		Number of deaths	
	Urban	Rural	Urban	Rural
Lung	25. 46	11. 37	1, 388	298
Larynx	2. 27	1. 25	124	33
Bladder	4. 13	2. 51	222	66
Tumors of the central nervous system	4. 61	3. 51	469	164

* Age adjusted on the basis of the total population of continental United States, 1950.

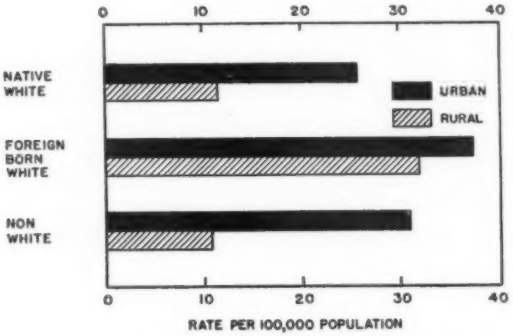
TABLE 10.—*Average annual age-adjusted death rate* due to cancer of the lung per 100,000 native white, foreign-born white, and nonwhite males, 25 to 64 years of age, residing in urban and rural areas of Ohio, 1947–51*

Color and nativity	Death rate		Number of deaths	
	Urban	Rural	Urban	Rural
Total	28. 28	12. 94	2, 090	372
White	27. 99	12. 99	1, 911	367
Native	25. 46	11. 37	1, 388	298
Foreign born	37. 30	32. 43	523	69
Nonwhite	30. 99	10. 75	179	5

*Age adjusted on the basis of the total population of continental United States, 1950.

on the other hand, was only 15 percent higher in urban than in rural areas. Another way of looking at the urban-rural differences in lung-cancer mortality is that the excess in mortality among the foreign-born white compared with the other two population subgroups was greater in rural than in urban areas. It may be noted that urban-rural comparisons based on death-certificate data present certain difficulties associated with allocation of residence at death.

In view of the urban-rural differences found in lung-cancer mortality a further exploration was made to test the validity of the observations by the use of comparisons of mortality in Census-defined Standard Metropolitan Areas with a population of 100,000 or more in 1950 and the rest of the State (table 11). The results showed a general similarity to those recorded in the above urban-rural comparisons. Age-adjusted lung-cancer mortality rates among the native white males and the nonwhite males 25 to 64 years of age were about 50 percent higher in Standard



TEXT-FIGURE 4.—Average annual age-adjusted death rate due to cancer of the lung among native white, foreign-born white, and nonwhite males, 25 to 64 years of age, residing in urban and rural areas of Ohio, 1947–51. (Age adjusted on the basis of the total population of continental U. S., 1950.)

Metropolitan Areas than in the rest of the State, while there was almost no difference between the age-adjusted mortality rates of the foreign-born white males, 25 to 64 years of age, in the two geographic subdivisions. Age breakdowns of the foreign born did, however, show some variation between Standard Metropolitan and other areas.

The 17 counties in the Standard Metropolitan Areas studied were further subdivided into two groups: the eight metropolitan counties with populations of 100,000 or more in 1950, and the nine "other counties" in the Standard Metropolitan Areas. It is of interest to note that the age-adjusted, lung-cancer mortality rate among the foreign-born white males, 25 to 64 years of age, was higher in the nine "other counties" than in the eight metropolitan counties, or in the 71 counties of the State located outside of the Standard Metropolitan Areas. The excess occurred particularly in the 25-to-44 year age group. On the other hand, the mortality rate among the native white males 25 to 64 years of age in the nine "other

TABLE 11.—*Average annual age-adjusted death rate* due to cancer of the lung per 100,000 native white, foreign-born white, and nonwhite male residents, 25 to 64 years of age, Standard Metropolitan Areas† and the balance of Ohio, 1947–51*

Geographic area	Color and nativity				
	Total	White	Native white	Foreign-born white	Nonwhite
	Death rate				
Ohio	23. 98	23. 59	20. 85	36. 67	29. 43
Counties in Standard Metropolitan Areas†	27. 11	26. 78	23. 77	36. 64	30. 10
Eight metropolitan counties	28. 48	28. 16	25. 47	35. 31	30. 96
Other nine counties	20. 04	19. 95	15. 69	45. 49	21. 99
Balance of State	16. 80	16. 71	15. 80	36. 93	21. 57
	Number of deaths				
Ohio	2, 462	2, 278	1, 686	592	184
Counties in Standard Metropolitan Areas†	1, 941	1, 768	1, 225	543	173
Eight metropolitan counties	1, 710	1, 549	1, 085	464	161
Other nine counties	231	219	140	79	12
Balance of State	521	510	461	49	11

*Age adjusted on the basis of the total population of continental United States, 1950.

†All Ohio counties in Standard Metropolitan Areas with a population of 100,000 or more in 1950 are included. These counties include the eight metropolitan counties shown on map, text-figure 1, plus Belmont, Butler, Clark, Greene, Jefferson, Lake, Lawrence, Lorain, and Trumbull. The Lima Standard Metropolitan Area (including Allen County) is the only Area excluded by the 100,000 population requirement.

counties" was considerably lower than in the eight metropolitan counties and, in fact, was similar to the mortality rate in the 71 counties located outside of the Standard Metropolitan Areas.

County Comparisons

Mortality due to each type of cancer studied showed considerable county variation when compared in each of the eight metropolitan counties of the State. The pattern of county variation differed by cancer site and population subgroup.

Individual county comparisons in mortality due to specific types of cancer among selected color and nativity groups of males 25 to 64 years of age are available in tables 12 and 13. A few of the highlights of the tables are as follows for each cancer site:

1) The number of lung-cancer deaths among native white males was higher than expected in Cuyahoga and Hamilton Counties. Mortality among foreign-born white males, on the other hand, was relatively high in Cuyahoga, Lucas, and Mahoning Counties and mortality among the nonwhite males was relatively high in Hamilton and Mahoning Counties. Observed lung-cancer deaths were lower than expected in Montgomery, Stark, and Summit Counties for each of the three population subgroups.

TABLE 12.—*Observed and expected* deaths due to cancer of the lung among native white, foreign-born white, and nonwhite male residents, 25 to 64 years of age, each of the eight metropolitan counties of Ohio, 1947–51*

County	Color and nativity				
	Total	White	Native white	Foreign-born white	Nonwhite
Eight metropolitan counties					
Observed deaths	1,710	1,549	1,085	464	161
Expected deaths	1,712	1,551	1,087	464	161
Cuyahoga					
Observed deaths	648	596	325	271	52
Expected deaths	606†	548†	290†	258	58
Franklin					
Observed deaths	163	146	134	12	17
Expected deaths	171	150	134	16	21
Hamilton					
Observed deaths	305	255	225	30	50
Expected deaths	261‡	227†	198†	29	34‡
Lucas					
Observed deaths	153	143	104	39	10
Expected deaths	157	147	114	33	10
Mahoning					
Observed deaths	108	93	42	51	15
Expected deaths	110	100	54	46	10
Montgomery					
Observed deaths	101	94	84	10	7
Expected deaths	135‡	121†	108†	13	14
Stark					
Observed deaths	89	87	71	16	2
Expected deaths	108	103	77	26	5
Summit					
Observed deaths	143	135	100	35	8
Expected deaths	164	155	112	43	9

*Deaths expected on the basis of the age, color, and nativity specific lung-cancer death rates among males in the combined eight metropolitan counties.

†Difference significant at $P = 0.05$ or less.

‡Difference significant at $P = 0.01$ or less.

2) Observed deaths due to cancer of the larynx were higher than expected in Cuyahoga and Lucas Counties among both the native white and foreign-born white males. There were some similarities in the county pattern of mortality for this site and for cancer of the lung.

3) Bladder-cancer mortality among the native white males was considerably higher than expected in Hamilton but was particularly low in Franklin and Montgomery Counties, while mortality due to this cause among the foreign-born white males was relatively low in Mahoning County.

4) Mortality due to tumors of the central nervous system among

the native white males showed a difference in county pattern from cancer of the lung or larynx in having the greatest excess of observed deaths over expected deaths in Summit County.

It should be pointed out that some of the differences noted are based on small numbers of deaths and are not statistically significant, as indicated in tables 12 and 13. It might also be noted that the tables pose statistical difficulties in multiple comparisons.

TABLE 13.—*Observed and expected* deaths due to cancer of selected sites among native white and foreign-born white male residents, 25 to 64 years of age, each of the eight metropolitan counties of Ohio: Cancer of the larynx and bladder, 1947–51; tumors of the central nervous system, 1944–52*

County	Cancer of larynx		Cancer of bladder		Tumors of central nervous system	
	Native white	Foreign-born white	Native white	Foreign-born white	Native white	Foreign-born white
Eight metropolitan counties						
Observed deaths	100	52	173	67	367	81
Expected deaths	100. 00	51. 90	172. 95	67. 05	366. 84	80. 91
Cuyahoga						
Observed deaths	35	32	50	39	98	44
Expected deaths	26. 60	28. 90	45. 15	37. 30	103. 59	44. 46
Franklin						
Observed deaths	13	1	9	4	39	5
Expected deaths	12. 35	1. 80	21. 50‡	2. 40	44. 64	3. 24
Hamilton						
Observed deaths	16	2	47	5	74	5
Expected deaths	18. 20	3. 25	32. 10‡	4. 10	63. 45	5. 58
Lucas						
Observed deaths	14	7	16	5	28	3
Expected deaths	10. 50	3. 65	18. 40	4. 70	37. 08	5. 67
Mahoning						
Observed deaths	5	5	8	2	15	5
Expected deaths	4. 95	5. 15	8. 35	6. 75	19. 35	7. 65
Montgomery						
Observed deaths	6	0	9	2	39	6
Expected deaths	10. 00	1. 45	17. 30†	1. 85	36. 09	2. 52
Stark						
Observed deaths	3	2	12	5	23	3
Expected deaths	7. 10	2. 90	12. 35	3. 70	25. 56	4. 50
Summit						
Observed deaths	8	3	22	5	51	10
Expected deaths	10. 30	4. 80	17. 80	6. 25	37. 08†	7. 29

*Deaths expected on the basis of the age, color, and nativity specific death rates for the specified cancer site among males in the combined eight metropolitan counties.

†Difference significant at $P = 0.05$ or less.

‡Difference significant at $P = 0.01$ or less.

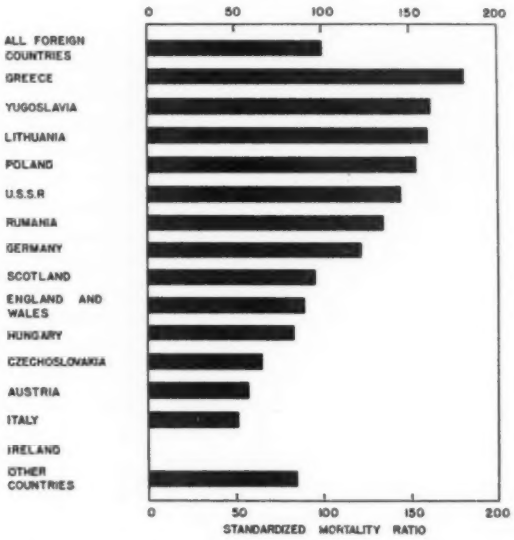
Country of Birth Comparisons
Cuyahoga County

Mortality among the foreign-born white males of Cuyahoga County showed marked variation by country of birth. The pattern of nativity variation differed among the cancer sites. In most instances, countries with relatively high mortality for one or more of the cancer sites showed lower mortality than expected for one or more of the other sites.

Table 14 brings out the nativity differences by comparisons of observed and expected deaths due to each type of cancer among Cuyahoga County male residents, 25 to 64 years of age, born in a number of European countries. It can be noted, for example, that lung-cancer mortality was higher than expected among males born in Poland, Yugoslavia, and the U.S.S.R. and particularly low among the Irish and Italian groups. Observed deaths due to cancer of the larynx were notably higher than expected among the Russians (*see also* text-fig. 5).

Mortality was higher than expected among the Yugoslavs for each cancer site and was consistently low among the Austrians. Generally, however, the differences between observed and expected deaths for these two nativity groups were based on small numbers of deaths and were not statistically significant.

It is also of interest to note that countries geographically near each other sometimes showed different mortality patterns. For example, although



TEXT-FIGURE 5.—Standardized mortality ratios of deaths due to cancer of the lung among foreign-born white male residents, 25 to 64 years of age, selected countries of birth, Cuyahoga County, Ohio, 1947–51. (Standardized mortality ratio = $\frac{\text{Observed deaths}}{\text{Expected deaths}} \times 100$. *See* table 14 for observed and expected death data.)

TABLE 14.—*Nativity comparisons in observed and expected* deaths due to cancer of selected sites among foreign-born white males, 25 to 64 years of age, residing in Cuyahoga County, Ohio: Cancer of the lung, larynx, and bladder, 1947–51; tumors of the central nervous system, 1944–52*

Country of birth	Cancer of the lung		Cancer of the larynx	
	Observed deaths	Expected deaths	Observed deaths	Expected deaths
All foreign countries	271	271.00	32	31.90
England and Wales	9	10.00	0	1.20
Scotland	5	5.15	0	0.65
Ireland	0†	6.30	1	0.75
Germany	17	14.00	1	1.70
Poland	59‡	38.60	6	4.40
Czechoslovakia	20	30.55	3	3.55
Austria	10	17.60	1	2.00
Hungary	26	30.75	3	3.50
Yugoslavia	29†	18.00	4	2.15
U. S. S. R.	29†	19.95	8‡	2.30
Lithuania	11	6.95	0	0.80
Rumania	6	4.45	1	0.45
Greece	7	3.85	1	0.50
Italy	18‡	35.20	3	4.20
Other countries	25	29.65	0	3.75

Country of birth	Cancer of the bladder		Tumors of the central nervous system	
	Observed deaths	Expected deaths	Observed deaths	Expected deaths
All foreign countries	39	38.95	44	43.83
England and Wales	0	1.45	2	1.80
Scotland	1	0.75	1	0.90
Ireland	1	0.90	1	1.08
Germany	2	2.05	5	2.43
Poland	4	5.65	5	5.85
Czechoslovakia	3	4.40	7	4.86
Austria	2	2.50	1	2.61
Hungary	6	4.30	5	4.68
Yugoslavia	6	2.60	3	2.79
U. S. S. R.	2	2.75	3	3.24
Lithuania	1	1.00	1	0.99
Rumania	0	0.60	2	0.72
Greece	0	0.55	1	0.63
Italy	5	4.95	4	5.76
Other countries	6	4.50	3	5.49

*Deaths expected on the basis of the age-specific mortality rates for the specified cancer site among all foreign-born white males in Cuyahoga County.

†Difference significant at $P = 0.05$ or less.

‡Difference significant at $P = 0.01$ or less.

lung-cancer mortality was especially low among the Italians it was higher than expected among the Greeks.

Age-adjusted, lung-cancer death rates were also computed for Cuyahoga County residents, 25 to 64 years of age, born in England and Wales and Italy for comparison with available data on mortality among the male population of the countries from which they migrated, as well as among native white males of Cuyahoga County. The results showed that the

mortality rate due to lung cancer among male immigrants to Cuyahoga County from England and Wales was similar to the death rate for this cause among the native white males of Cuyahoga County but considerably lower than among the male population of England and Wales. On the other hand, the lung-cancer mortality rate among male immigrants from Italy to Cuyahoga County was similar to the male lung-cancer mortality rate in Italy but considerably lower than among native white males of Cuyahoga County. The age-adjusted, lung-cancer death rates [4] per 100,000 males, 25 to 64 years of age, were as follows for the different population groups:

Population of	*Death rate*	*Immigrants to Cuyahoga County from*	*Death rate*
Cuyahoga County, 1947–51 (Native white)	28. 75	All foreign countries, 1947–51	38. 11
England and Wales, 1950	55. 48	England and Wales, 1947–51	31. 75
Italy, 1951	16. 26	Italy, 1947–51	18. 61

It is of interest to compare the findings for Cuyahoga County with the results of the study by Eastcott of cancer mortality among native and immigrant groups in New Zealand (*3*). As noted earlier, Eastcott found considerably higher lung-cancer mortality among immigrants born in the United Kingdom than among the native New Zealand population. On the other hand, as noted above, data for Cuyahoga County showed similar lung-cancer mortality rates among male immigrants from England and Wales and native white males. This probably reflects in part higher lung-cancer mortality rates among native born males 25 to 64 years of age for Cuyahoga County than for New Zealand during the period 1947–51.

In interpreting the nativity differences in cancer mortality it is important to remember the possible limitations of the reported mortality and population data due to boundary changes in Europe, as noted earlier in the paper. There is, however, no reason to expect any possible influence of the boundary changes to differ among the cancer sites, except that deaths due to tumors of the central nervous system occurred among younger age groups. As indicated above, the mortality data for individual nativity groups do show considerable difference by site.

Duration of Residence

Available residence history data for white male residents of the eight metropolitan counties who died of histologically confirmed tumors of the central nervous system from ages 25 through 64 indicate considerable stability of residence.

Table 15 provides residence data for the metropolitan counties for native and foreign-born white males, classified by country of birth, who died of tumors of the central nervous system. The data show the number of years of residence in the county of residence at death. The period of residence represents consecutive years prior to death and includes only the years in which place of residence was confirmed. The table brings out the fact that the majority of the people had lived for a considerable

[4] Age adjusted on the basis of the total population of continental United States, 1950. Death rates for England and Wales, and Italy are based on data used for table 6.

TABLE 15.—*Duration of residence in county of residence at death of native and selected foreign-born groups of white males who died of tumors of the central nervous system, ages 25 to 64 years at death, eight metropolitan counties of Ohio, 1944–52*

Nativity	Years of residence prior to death*						
	Total	Under 5	5–9	10–14	15–19	20 and over	Unknown
	Number of persons						
Native white	367	7	38	43	34	215	30
Foreign-born white	81	1	8	11	7	45	9
England and Wales	7	—	1	—	2	3	1
Germany	10	—	1	2	1	6	—
Poland	10	—	1	1	1	7	—
Czechoslovakia	9	—	1	1	1	6	—
Austria and Rumania	6	—	1	—	—	5	—
Hungary	5	—	—	1	—	2	2
Yugoslavia	3	—	1	1	—	1	—
U. S. S. R.	4	—	—	—	2	1	1
Lithuania	1	—	—	—	—	—	1
Greece	5	—	—	2	—	3	—
Italy	13	—	1	3	—	8	1
Other	8	1	1	—	—	3	3

*Includes only consecutive years of residence before death.

period of time in the county in which they resided at death. Only 7 of the 337 native white males and only 1 of the 72 foreign-born white males for whom residence data were established had lived less than 5 years in the county of residence at death.

Discussion

The findings of this study concerning the foreign born raise a basic question: Are the nativity differences in cancer mortality due to factors associated with the place an individual is born or the place to which he migrates? Some of the factors to consider in this basic question are:

1) Inherent characteristics of the population of various racial stocks in the country of birth.
2) Cultural, socio-economic, or other environmental circumstances of the country of birth, and the geographic area within the country.
3) Selective and motivating factors responsible for migration.
4) Cultural, socio-economic, or other environmental characteristics of the state, county, city, and local subarea of the country into which an individual migrates, and the length of residence there.

The present study makes possible only limited comment on the basic question raised. It can be noted in reference to country of birth that other writers, such as Steiner and Hueper, have raised similar questions regarding the carcinogenic importance of events in early life and the early *versus* the late effects of environmental factors in cancer frequency (*4, 18*). A point of interest with reference to conditions following migration is the

tendency observed for specific nativity groups to settle in particular areas in which they often live and work together, possibly associating with a specific industry or occupation group, and maintain the cultural characteristics of their nativity group. The long latent period before cancer appears makes it necessary to consider factors in both the country of birth and the place to which migration occurred in terms of circumstances which prevailed as long as 20 or 30 years or more ago.

Previous investigators have noted an association between socio-economic status and cancer (*19, 20*). An earlier report on cancer in Ohio indicated that in Cuyahoga County, high lung-cancer mortality among white males was associated with low economic status, low educational level, and foreign birth (*1*). It would appear to be advisable to make further explorations of any interrelationships of these three factors. A specific characteristic requiring study is industry and occupation of the different nativity groups. The long latent period in cancer mortality makes life industrial histories advisable to detect any possible environmental influence. Sex and residence comparisons of population subgroups studied would be desirable for further defining the mortality differences and any factors associated with them.

In recent years writers have focused attention on urban-rural differences in mortality due to cancer of specific sites. The Ohio study further delineates the urban-rural differences through consideration of additional population subgroups. A point of particular interest is the finding that lung-cancer mortality among native white males was considerably higher in urban than in rural areas. On the other hand, the lung-cancer mortality rate among the foreign-born white males, which was higher than among the native white males in urban areas, did not drop appreciably in rural areas. These findings suggest some factors in urban and rural areas affecting the native white male lung-cancer mortality rate. On the other hand, there appear to be possible common factors affecting the foreign-born male lung-cancer mortality rates of both urban and rural areas. Additional exploration of mortality rates by specific country-of-birth groups for urban and rural areas would be advisable in order to delineate the nature of the foreign-born mortality in the two areas. This was not possible in the present study because of lack of necessary population data.

The findings presented suggest that in the near future cancer mortality studies include nativity identifications of the population so the extent of the foreign-born influence on cancer mortality rates can be determined. This is especially important in the older age groups in which the foreign born are concentrated. For example, in the present study for Ohio the lung-cancer mortality rate among the native white males was approximately 15 percent lower than among the total white males for the age group 55 to 64. Such an identification of the native and foreign-born white population groups is important in comparisons of nonwhite and white population groups.

Further investigation of foreign-born cancer mortality in other areas of the country might be considered to provide comparative data. Such

studies might include the same nativity groups considered in Ohio as well as other nativity groups not included in the study, such as the Swedish in Minnesota and the Chinese and Japanese in California.

The considerations noted above indicate the importance of adequate population statistics on the foreign born by age, sex, nativity, and local residence for use in mortality studies. It would appear to be desirable to have the statistical tabulations by the Bureau of the Census made in a form to meet this need.

The concentration of the foreign born in the older age groups is of special importance for future cancer mortality studies for two reasons. First, cancer mortality is relatively high in the older age groups. Second, it means that if past migration trends prevail, studies of the foreign born must be initiated in the near future if they are to be made at all.

Finally, the nativity differences of this study point out the need to consider social and cultural characteristics of an area as possible epidemiological leads for factors associated with cancer of specific sites.

Summary

This paper compared mortality due to cancer of the lung, larynx, bladder, and tumors of the central nervous system among native white, foreign-born white, and nonwhite male residents of Ohio 25 to 64 years of age. Briefly the findings are as follows for the four cancer sites studied:

1) Mortality was higher among the foreign-born white than among the native white males for each cancer site. Mortality among the nonwhite males was intermediate between mortality for the two white male population subgroups for cancer of the lung. On the other hand, the nonwhites had an age-adjusted mortality rate similar to the foreign-born whites for cancer of the larynx, similar to the native whites for cancer of the bladder, and lower than either of the white population subgroups for tumors of the central nervous system.

2) Mortality increased with age for each of the three population subgroups for each cancer site. The excess in mortality among the foreign-born white males compared with the native white males was highest at ages 55 to 64 for cancer of the lung and at ages 25 to 44 for tumors of the central nervous system.

3) Urban-rural comparisons in lung-cancer mortality, based on the 1950 Census definition of urban and rural areas, showed a marked urban excess for the native white as well as the nonwhite population group. In comparison, the lung-cancer mortality rate among the foreign born, which was higher than for either of the other population groups in urban areas, did not drop appreciably in rural areas. In fact, the age-adjusted mortality rate among the foreign born of rural areas was similar to the rate of the nonwhite of urban areas and somewhat above the rate for the native white of urban areas.

4) Mortality varied among each of the eight metropolitan counties of the State for selected population subgroups studied. The pattern of county variation differed by cancer site and population subgroup.

5) Mortality of the foreign-born white male residents of Cuyahoga County varied considerably by country of birth. For example, lung-cancer mortality was higher than expected among males born in Poland, Yugoslavia, and the U.S.S.R. and relatively low among Irish and Italian males. Mortality due to cancer of the larynx was particularly high among males born in the U.S.S.R. In the 25-to-64 year age group the age-adjusted, lung-cancer mortality rate among male immigrants to Cuyahoga County from Italy was similar to mortality due to this cause among the male population of Italy but considerably lower than among native white male residents of Cuyahoga County. On the other hand, the lung-cancer mortality rate among male immigrants to Cuyahoga County from England and Wales was similar to the mortality rate due to this cause among native white males of Cuyahoga County but was considerably lower than among the male population of England and Wales.

6) Residence histories of native and foreign-born white males who died of tumors of the central nervous system showed that they had lived for a considerable period of time in the county in which they resided at death.

In conclusion, the findings bring out the importance of identifying nativity as well as residence in cancer mortality statistics.

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Epithelial Tubule Formation by Mouse Metanephrogenic Mesenchyme Transplanted *in Vivo* [1]

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A number of descriptive and experimental studies (*1*) have demonstrated the general dependence of epithelial tubule formation by nephrogenic mesenchyme upon association with the wolffian duct or its derivative ureteric bud. In the mouse, both certain genetic abnormalities (*2*) and the behavior *in vitro* of metanephrogenic rudiments and their components (*3*) indicate such dependency. The observations *in vitro* have confirmed earlier reports (*4*) that embryonic spinal cord is an effective inductor of kidney tubules, and some information has been obtained on the nature and mode of transmission of the inductive influence (*5–7*).

Besides the abundance of data indicating that kidney tubules can form by induction, there are frequent reports of the formation of epithelial tubules in nephrogenic mesenchyme in the absence of an identifiable inductive source (*1*). Such "spontaneous" formation, or "self-differentiation," of kidney tubules is paralleled in other inductive systems, *e.g.*, "free lens" formation (*8*) or neural differentiation of gastrular ectoderm *in vivo* in the absence of chordamesoderm (*9*). These deviations from expectation on the basis of current theory have been rationalized as self-induction, precocious induction, double assurance, or the result of the widespread occurrence of unknown inductors. The last-named has been invoked particularly in connection with self-differentiation in various adult transplantation sites. These have been avoided by most investigators on the reasonable assumption that the action of "true" inductors operative in embryogenesis might be obscured by unrelated inductors in the adult.

The problem arose in current studies when metanephrogenic mesenchyme was transplanted to the anterior chamber of the eye after exposure of increasing duration *in vitro* to dorsal spinal cord. This effort to determine minimum induction time was balked by the finding that the mesenchyme would form tubules without exposure to spinal cord, both on direct implantation and following periods in culture up to about 3 days. Beyond this time, tubule formation on implantation was progres-

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447911—58——12

sively weaker, adipose tissue becoming the predominant or exclusive differentiation. Two problems were thereby raised: 1) the nature of the "*in vivo* effect," and 2) the nature of the change in metanephrogenic mesenchyme during culture, which alters its differentiative response. The experiments described below bear on the first problem. They show that metanephrogenic mesenchyme, without its normal inductor, can form tubules not only in the eye but in other *in vivo* sites; that in the eye, direct tissue contact is not required, and that whatever is involved *in vivo* is not transferable to culture conditions by methods so far used.

Materials and Methods

Metanephrogenic rudiments were dissected from 11-day embryos, obtained from BALB/c females impregnated by C3H males (C × C3H). All mouse hosts in implant experiments were C × C3H males from 10 to 18 weeks of age. The kidney rudiments varied somewhat in stage, the size and shape of the ureteric bud ranging from a simple, slightly flattened bulb to a broadly flattened but nonbranching "pelvis". The procedure for separating the rudiment components in trypsin solution has been described (*3*). The term "metanephrogenic mesenchyme" will be used to designate the whole mesenchymal component of the rudiment, following complete removal of the wolffian duct and ureteric bud. Unless otherwise specified, all implants and explants consisted of mesenchyme from a single rudiment.

The technique for intraocular transplantation has been described (*10*). A single implant was made into the right eye of each host animal. When an effort was made to avoid contact between the implant and the iris, partial iridectomy was performed. Following a shallow corneal incision, the unperforated iris frequently protrudes. The projecting blister was seized at the base with forceps and pulled sharply, which usually removed, reasonably clean, from a third to more than a half of the iris. The implant was then placed at the center of the iridectomized portion of the eye.

The implants were examined daily *in situ* during critical periods, and size, color, position, evidence of vascularization, etc., were recorded. At appropriate intervals, the hosts were killed by cervical dislocation, and the eye was quickly snipped out and dropped into Zenker-acetic. Subsequently, during dehydration, the eye was opened, the lens removed, and the implant identified. When the implant could not be identified, the entire eye was sectioned after removal of the lens. Other transplant procedures will be described briefly.

Culture methods used have been described (*3*). The nutrient medium consisted of horse serum, Tyrode's solution and chick-embryo juice (2:2:1), and was changed daily. Penicillin, streptomycin, and mycostatin were incorporated in all culture media at concentrations recommended by the manufacturers. Clots were made with adult fowl plasma and nutrient medium (1:1). All cultures were incubated at 37.5° C. in an atmosphere of 5 percent CO_2 saturated with water vapor.

Results

Intraocular implantation of metanephrogenic rudiments and their components.—To test the intraocular behavior of intact metanephrogenic rudiments and each of their components, 7 pairs of rudiments were removed from 11-day embryos of the same litter. One of each pair was treated with trypsin and each of the components was separately implanted. The other member of the pair was similarly treated with trypsin but implanted intact. The implants were examined *in situ* on the tenth day and again on the 21st day when they were fixed for serial sectioning.

None of the ureteric bud implants were identified in the living animal or sections. The isolated epithelium either failed to survive or was too inconspicuous to be recognized. All but two of the mesenchymal implants were observed in the eye where they ranged in area from 0.2 to 1.0 mm.^{2}, and all were found in sections. Each had formed clearly recognizable epithelial tubules, and renal capsules and glomeruli were identifiable in most (fig. 4). Similarly, all of the intact rudiments were found. All showed epithelial tubules, and all but one possessed capsules and glomeruli (fig. 1). The four largest (0.6 to 3.5 mm.^{2}) contained collecting tubules derived from the original epithelial component. Three others, which were either small in the living eye or indistinguishable, were without collecting tubules and resembled the grafts of mesenchyme alone.

The results indicate that: 1) metanephrogenic epithelium is dependent on its mesenchymal association for continued development in the eye; 2) metanephrogenic mesenchyme can form tubules and glomeruli in the eye in the absence of its epithelium, though the development of intact rudiments is more vigorous, presumably because of the interaction of mesenchyme and the epithelial ureteric bud.

Early interaction of metanephrogenic mesenchyme and iris.—To observe the early stages of implantation and tubule formation, metanephrogenic mesenchyme was implanted into the eyes of 45 adult male mice. The animals were killed from 1 to 7 days later and the graft area serially sectioned. It is clear from table 1 that epithelial tubules are formed in the eye on the third and fourth days, the exact time presumably varying in individual cases with the favorableness of the graft placement. In the most favorable circumstances tubule formation was obvious by the third

TABLE 1.—*Tubule formation in the eye by metanephrogenic mesenchyme*

Days in eye	No. of cases	Definite tubules	Early or weak tubules	No tubules
1	4	0	0	4
2	3	0	0	3
3	13	3	5	5
4	8	4	2	2
5	8	8	0	0
6	6	6	0	0
7	3	3	0	0

day; 3 of 13 cases fixed at this time already possessed well-developed tubules (fig. 3).

Vigorous interaction between the iris and the implanted mesenchyme in the eye marked the first several days. This was apparent by 24 hours (fig. 2) when the implant in all 4 cases was found to lie between, and in contact with, both iris and cornea. The iris was spongy in appearance with strands of pigmented cells projecting into the mesenchyme and, frequently, partially isolating small nests of mesenchymal cells. In 3 of the 4 cases iridial blood vessels could be traced into the graft, and in these cases large pools of red cells were interspersed in the graft tissue, correlated with bright-red color in the living state. The fourth graft was white in the living eye and showed no evidence of vascularization in sections.

Vascularization and interpenetration of graft and iris were more advanced in the three 48-hour cases. In places the continuity of the iris was interrupted, as mesenchyme had penetrated to the posterior chamber side. The thirteen 3-day cases were variable, not only in the occurrence of tubules but in the relations of the graft to the iris and cornea. Tubules, when present, were always close to the iris or, more usually, actually embedded in it. In 8 cases the graft was not in contact, or only partially in contact, with the cornea. Portions of grafts associated with the cornea almost always differed in appearance from those associated with the iris in being less condensed and basophilic. This is particularly clear in one of the eight 4-day cases, which had been observed daily in the living state and had remained white, suggesting that it had not come into contact with the iris or become vascularized (fig. 6). In sections, no tubules were present and the tissue showed a gradient of basophilia from iridial to corneal sides.

Characteristically at 5 days there was no contact, or much reduced contact, between graft and cornea. Since less and less contact was seen in later stages, it may be assumed that at about this stage affinity of the graft for the cornea either declines or the graft is pulled free by its closer affinity for the iris. With one exception, grafts still in contact with both iris and cornea possessed tubules in the iridial portion but not in the corneal portion (fig. 5). The exception involved a single tubule rudiment in a tongue of graft tissue projecting along the corneal surface.

Tubule formation without contact with the iris.—The preceding observations suggested that contact with the iris might be essential for tubule formation by metanephrogenic mesenchyme in the eye. To test this, the procedure was varied in an effort to prevent the implants from coming into contact with the iris. Variations consisted of implantation into partially iridectomized eyes, or of implantation of small fragments that might float freely or adhere to other structures than the iris. Most of the 77 implants were unsuccessful, *i.e.*, the implants usually contacted the iris in whole or in part and formed tubules in the preponderance of cases. However, 6 were adherent to, or spread on, the cornea, and of these only 2 formed tubules. Four out of 5 implants adherent to, or spread on, the lens surface formed tubules. And all 5 implants found floating in the

anterior chamber, with minimal or no contact with other structures, showed tubule organization, though with minimal differentiation (fig. 7). In the case illustrated, the graft had been examined *in situ* on the second day and recorded as floating in the pupil, without apparent contact. On the fifth day, when it was fixed, it was observed as a small rounded bead just to one side of the pupil. There was never any sign of vascularization or contact with the iris.

It seems safe to conclude that tubule formation can be initiated in the eye without contact with the iris when the graft is free-floating or in contact with the lens. Contact with the cornea may tend to inhibit tubule formation, and contact with the iris, while not essential, may promote growth and differentiation through vascularization, or in other ways.

Implants into adult brain.—Eight intact metanephrogenic rudiments and 10 implants of metanephrogenic mesenchyme were made into the brain of adult male mice. Each implant of mesenchyme separated from epithelium was multiple and derived from 3 rudiments. The implantation procedure was essentially that described by Greene (*11*). A small chisel was fabricated and used to cut through and lift a skull flap. The tissue was introduced into the brain with a trocar made from a 20-gauge hypodermic needle, and the wound was subsequently sealed with collodion. The animals were killed at 10 to 12 days and the brain fixed for serial sectioning.

In all but one case the implant was identifiable in the sections. All of the intact rudiments formed healthy masses with clearly identifiable collecting ducts as well as secretory tubules and glomeruli. The behavior of the metanephrogenic mesenchyme was more variable both in extent of growth and tubule formation. In all but two cases, however, differentiation of epithelial tubules was clearly identifiable (fig. 9) and glomeruli usually were present as well. It may be concluded that tubule formation occurs in metanephrogenic mesenchyme implanted into adult brain.

Subcutaneous implants.—In 9 experiments subcutaneous grafts were made of intact rudiments or of metanephrogenic mesenchyme alone. Graft recovery was poor in most instances. In one experiment, however, the procedure described by Merwin and Hill (*12*) was used and better success attained. The procedure involves production of a dorsal skin fold, incision through one side, and placement of the graft in the subcutaneous tissue of the opposite side. When the fold is released the graft comes to lie at a considerable distance from the incision. To increase the size of the graft, metanephrogenic mesenchyme from 5 rudiments was fused into a single mass by culturing at 37° C. for about 20 hours. Six such implants were made along with 6 single intact rudiments. All of the intact rudiments were recovered after 10 to 12 days, each showing definite tubular differentiation and 2 being well vascularized and quite large. Five of the 6 mesenchymal grafts were recovered. All were small, but 2 had formed sparse and weakly differentiated epithelial tubules (fig. 10). It may be concluded that the subcutaneous site is not

favorable for survival and differentiation of metanephrogenic mesenchyme, but it appears that when minimum survival requirements are met, epithelial tubule formation can occur.

Grafts to the chick chorio-allantoic membrane.—Forty grafts in 6 experiments were made to the chorio-allantoic membrane of the 8-day chick embryo. Metanephrogenic mesenchyme from 2 to 8 rudiments were first combined into a single mass during a 20-hour culture period, and then transferred to the chick membrane for 4 to 8 days. Included among the grafts were 6 single intact rudiments. One of these was recovered as a small vascularized mass with epithelial tubules. Probable viable graft tissue was identifiable in 13 out of 34 grafts of metanephrogenic mesenchyme, usually in explants involving larger numbers of rudiments. Epithelial tubules were not observed in any case. It may be concluded that the chorio-allantoic membrane is not a favorable site for survival and growth of metanephrogenic rudiments. There is, however, no evidence that epithelial tubules form in metanephrogenic mesenchyme even when significant survival of the graft occurs.

Intracoelomic grafts in the chick embryo.—Survival and epithelial tubule formation is much more readily obtained in grafts to the coelomic cavity of approximately 65-hour chick embryos. Following the procedure of Hamburger (*13*), 11 grafts were made into the anterior coelomic cavity. After 5 days the embryos were fixed, the head and posterior portions removed, and the remainder sectioned serially. Six of the grafts were intact 11-day kidney rudiments precultured for 20 hours. All were identifiable in the sections, both by their ectopic position and their distinctly more basophilic staining as compared with the surrounding chick tissues. All had formed epithelial tubules. Similarly, 5 grafts were made of metanephrogenic mesenchyme, each including tissue from 3 rudiments fused during a 20-hour culture period. Four of these were identified in the sections, each containing distinct epithelial tubules and occasional glomeruli. Two were in the body wall in the region of the brachial plexus (fig. 8), and 2 were in the region of the chick mesonephros and readily distinguishable from it. It may be concluded that metanephrogenic mesenchyme can form epithelial tubules in the chick embryo between the third and eighth day of incubation.

In vitro *tests for inductive activity of adult mouse tissues and fluids.*—Possible inductive effects of tissues and fluids from the mouse eye and brain *in vitro* were tested in a series of experiments. Metanephrogenic mesenchyme was combined in culture with the tissues to be tested by procedures earlier found to give positive results with embryonic dorsal spinal cord (*3*). Thus, in 4 cases pieces of iris were placed at the glass-clot interface in the center of a cluster of mesenchyme pieces, in 2 cases the mesenchyme was surrounded by iris under similar circumstances, and in 2 cases the mesenchyme was placed on top of iris spread on the surface of a clot. The tissues fused into a single mass in all instances, but epithelial tubules formed in none.

In the same way, adult cornea (3 cases), adult retina (3 cases), and

adult brain (cerebral cortex, hypothalamic region, corpus callosum, 6 cases each) gave negative results when placed at the center of a cluster of mesenchyme pieces or when arranged in a cluster around a mesenchyme piece.

Intraocular fluids were tested in a total of 13 cultures, in an effort to simulate the conditions under which floating grafts produced tubules in the eye. In one experiment, intraocular fluid was prepared by removing from 6 male mice 12 eyes similar to those used for grafting. The eyes were washed briefly in Tyrode's solution, blotted to remove adhering fluid, and cut open with scissors. The fluids were collected, transferred to a small tube, and centrifuged lightly to remove tissue fragments. One third of the cloudy supernatant was diluted with an equal volume of culture medium (IM), the remainder was used without dilution (I). A similar amount of culture medium (M) was used as control. Three cultures were prepared with each medium by suspending a drop below a filter-ring assembly (*5*) and placing metanephrogenic mesenchyme in the drop so it came to rest at the bottom. Contact of the explant with a solid substrate was avoided, the explant rounding up as a spherical mass. Most of the medium was withdrawn daily and replaced by a freshly prepared drop. The cultures were incubated for 5 days. Following fixation and sectioning, epithelial tubules were not found in tissues cultured in any of the media. The explants were mainly healthy in M or IM and less so in I where viable cells were limited to the peripheral region. There was no evidence of any behavior comparable to that observed in the eye.

Discussion

It may be concluded from the data described that mouse metanephrogenic mesenchyme, separated from its normal inductor, can form epithelial tubules in several sites *in vivo*. Whatever is involved *in vivo* is not readily transferred to conditions *in vitro*. Cultured metanephrogenic mesenchyme does not produce epithelial tubules in tissues or fluids derived from the active *in vivo* sites under conditions which allow epithelial tubules to be induced by several embryonic inductive sources. The "*in vivo* effect" does not depend upon tissue contact since it occurs in implants freely floating in intraocular fluid. And it is not either tissue specific or species specific, since it occurs not only in the eye but also in the adult mouse brain and in the chick embryo.

These puzzling facts, as noted above, have their parallel in other inductive systems. Under proper conditions in some species, lens formation occurs *in vivo* in the absence of the optic vesicle, though the same epithelium does not form lens *in vitro* (*14*). Amphibian gastrular ectoderm, which fails to neuralize under proper conditions in culture, forms nervous tissue when floating freely in the larval coelom (*9*). Indeed, there is evidence that gastrular ectoderm not only undergoes more complex differentiation *in vivo* than *in vitro*, but that its differentiation varies qualitatively depending upon the site of transplantation (*15*). Earlier in the study of inductive phenomena these differentiations *in vivo* were regarded as an

indication of the general systemic occurrence of inductors, and effort was concentrated on developing a "neutral" environment. With the demonstration of "self-neuralization" *in vitro* and of differential inductive effects of various killed adult tissues (*16*), the nature of the *in vivo* effect deserves further study.

Unfortunately, the data recorded here re-emphasize the problem but contribute little to its solution. Three general possibilities exist. First, metanephrogenic mesenchyme may require a specific extrinsic contribution that is found in many places, and particularly abundantly in certain embryonic tissues. The inducing material must then be thought of as sufficiently unstable to be demonstrable *in vitro* only when present or produced in the relatively large local concentrations characteristic of embryonic inductive sources. Second, metanephrogenic mesenchyme may require no further positive extrinsic contribution being able to "self-differentiate" unless inhibited by unfavorable conditions such as exist *in vitro*. In this event, the normal contribution of ureteric bud must be thought of as already made at the stage under consideration, or its further contribution merely protects against disruption of properties already present. In these terms the "*in vivo* effect" is likewise a "sparing" rather than a positive one.

Third, making the minimum of assumptions about the nature and mechanism of the factors involved, epithelial tubule formation may be thought of as imminent in the metanephrogenic mesenchyme in a probability sense. *In situ*, under the conditions resulting from a series of prior developmental steps undergone by metanephrogenic mesenchyme, the presence or absence of ureteric bud is determinative. But under the very different conditions of culture or transplantation, other factors may enter as determinants, raising or lowering the probability of tubule formation. In this case, the *in vivo* effect, which appears to operate on several inductive systems, may be a general factor favoring differentation rather than a specific one for metanephrogenic mesenchyme. It may, for example, be related to the factor whose absence accounts for the generally poor differentiation of tissues *in vitro*. Further elucidation of the mechanism of action of this and other influences that raise or lower the probability of tubule formation by metanephrogenic mesenchyme may contribute to an understanding of the state of developmental imminence or, as it has been called in the case of the reactive component of inductive systems, "competence."

Summary

Metanephrogenic mesenchyme from 11-day mouse embryos forms epithelial tubules when implanted without ureteric bud into such sites as the anterior chamber of the eye, brain, or subcutaneous tissue of the adult mouse, or the body cavity of the 4-day chick embryo. Epithelial tubules are not formed on the chick chorio-allantoic membrane. Tubule formation does not depend on tissue contact in the eye since it occurs in implants freely floating in the anterior chamber. The *in vivo* behavior contrasts sharply with that *in vitro* where tubule formation only occurs in close association with one of several embryonic inductors and is not

induced by tissues or fluids from the active *in vivo* sites tested here. The nature of the *in vivo* effect is not clear and deserves investigation for its bearing on differentiative mechanisms.

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PLATE 9

FIGURE 1.—Intact kidney rudiment after 21 days in the eye. *Note* crescent-shaped section through collecting duct, numerous glomeruli. × 110

FIGURE 2.—Metanephrogenic mesenchyme after 1 day in the eye. *Note* interpenetration of iris and mesenchyme. × 170

FIGURE 3.—Metanephrogenic mesenchyme after 3 days in the eye. *Note* epithelia tubule embedded in spongy iris. × 170

FIGURE 4.—Metanephrogenic mesenchyme after 21 days in the eye. *Note* glomerulus and tubules. × 110

FIGURE 5.—Metanephrogenic mesenchyme after 5 days in the eye. *Note* tubule formation in association with the iris, not with cornea. × 170

FIGURE 6.—Metanephrogenic mesenchyme attached to cornea after 4 days in the eye. No tubule formation. × 170

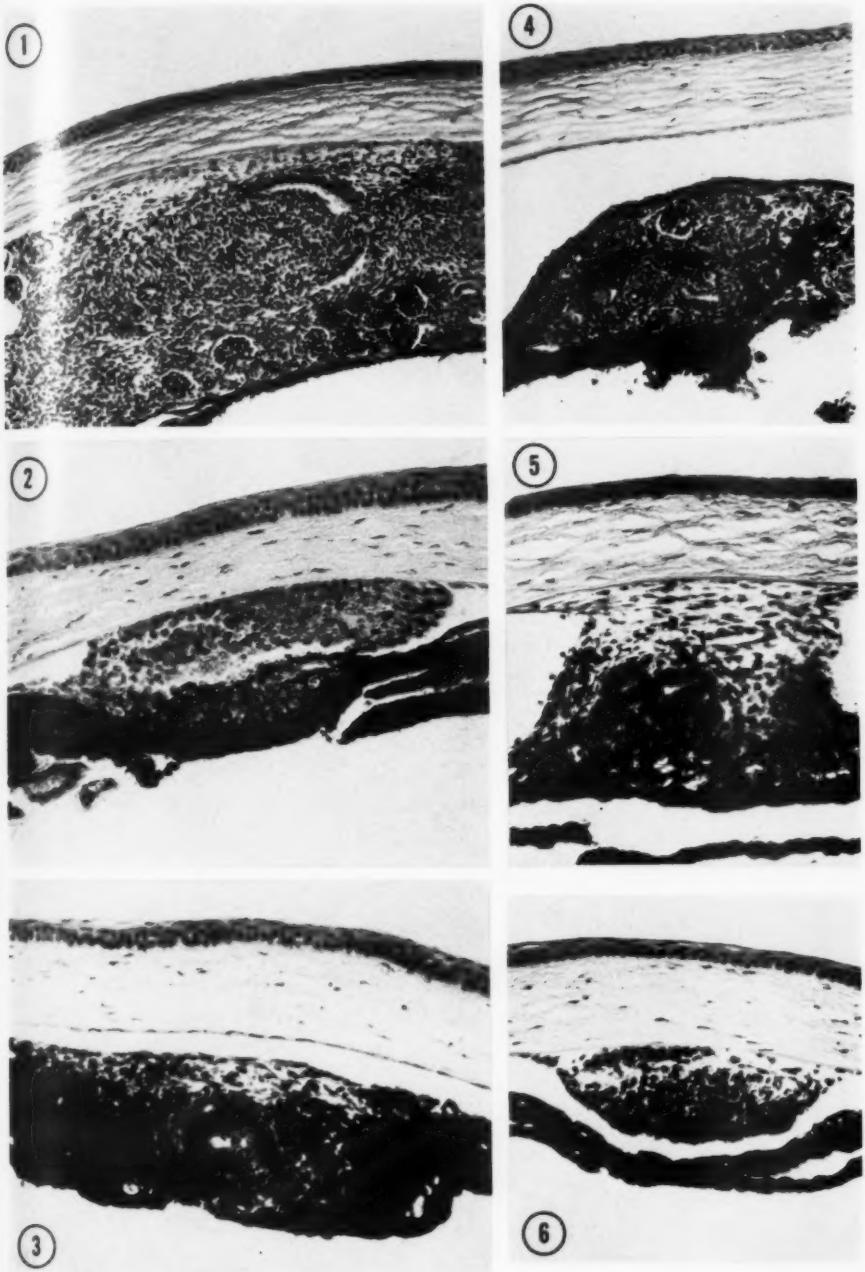

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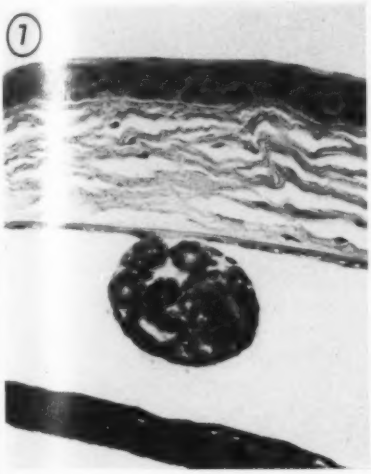
PLATE 10

FIGURE 7.—Metanephrogenic mesenchyme after 6 days in the eye. Tubular organization has occurred in implant floating freely except for small contact area with cornea. × 240

FIGURE 8.—Mouse epithelial tubules formed in the region of the brachial plexus of a chick embryo after intracoelomic implantation of metanephrogenic mesenchyme. × 120

FIGURE 9.—Epithelial tubules formed from metanephrogenic mesenchyme implanted into the brain of an adult male mouse. × 170

FIGURE 10.—Weak tubule formation from implanted metanephrogenic mesenchyme in the subcutaneous muscle layer of an adult male mouse. × 170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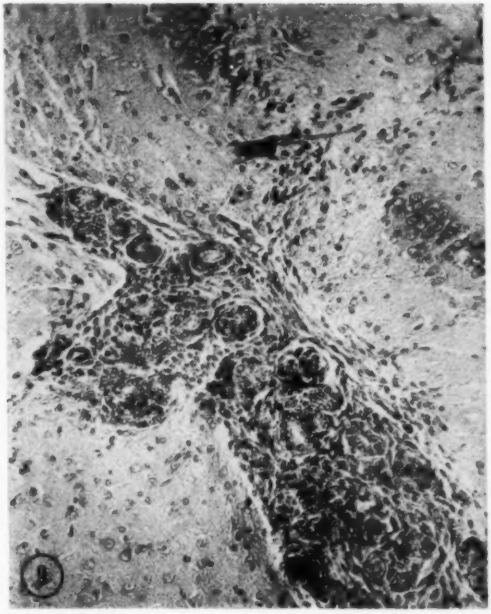
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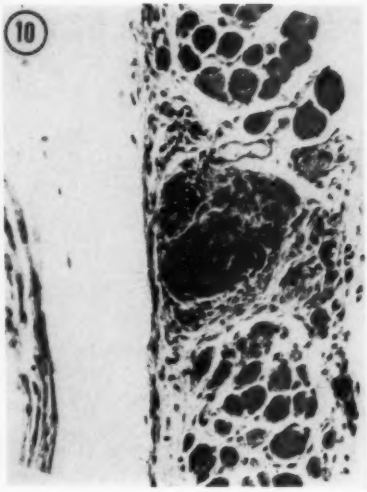
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Studies on the Difference in Sarcoma-Producing Capacity of Two Lines of Mouse Cells Derived *in Vitro* from One Cell [1]

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In previous studies (*1–3*), strains of cells from subcutaneous and muscle tissues of the mouse were found, after culture *in vitro*, to differ in their capacity to grow as sarcomas on implantation in mice of the strain of origin. These differences in sarcoma-producing capacity could conceivably have resulted from different degrees or types of malignant alteration occurring in the cells during culture or from different antigenic changes in the cells that influenced their transplantability.

The three studies reported in this paper were undertaken to explain the difference in sarcoma-producing capacity exhibited by two lines of mouse cells derived *in vitro* from one cell (*3*). The cell had been isolated from a culture of subcutaneous adipose tissue removed from the flank of an adult mouse of strain C3H. From the derived clone culture, lines of cells were established that underwent both morphologic and physiologic transformations during the first 18 months of growth *in vitro*. Two of these lines, which differed widely in sarcoma-producing capacity, were continued for a more intensive study of their characteristics. The cells of one line designated high sarcoma-producing, or "high," produced sarcomas in 97 percent of the mice injected. The cells of the other line designated low sarcoma-producing, or "low," [3] produced sarcomas in only 1 percent of the mice, unless the animals had previously been X-irradiated; under this condition almost half the mice developed sarcomas.

The increased frequency of tumors from low-line cells in irradiated mice suggested that the cells of this line might be immunologically incompatible with the host since irradiation is known to impair the development of immunity to incompatible cells and to allow their growth *in vivo* (*4*). An immunologic incompatibility was demonstrated previously between another strain of tissue-culture cells and mice of the strain from which the cells originated (*5*). The first study reported in this paper considered the possibility that cells of the low line might differ from

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] In the previous study (*3*) these two lines were referred to as VII and III, respectively. As now carried in this laboratory they are designated NCTC strain 1742 (high line) and NCTC strain 2049 (low line).

those of the high in being antigenic to mice of the strain of origin (C3H). It was found that cells of both lines were antigenic to mice of strain C3H and that the two cell lines produced cross-immunity in mice of this strain.

A second possibility was then considered: Cells of the high sarcoma-producing line might differ from those of the low in producing an established tumor before immunity developed in the host. It is known that after a tumor transplant is established in a resistant host, acquired immunity may be ineffective in causing regression (*6*). The second study determined: 1) the time required for the development of palpable tumors from known numbers of cells of each line in nonirradiated and X-irradiated (C3H) mice; 2) the interval before acquired homologous immunity developed in these mice, since no accurate method is available for measuring the period required for development of immunity to the tissue-culture cells of isologous origin. It was found that in X-irradiated hosts, cells of the high line did produce palpable tumors before detectable homologous immunity developed in the host, whereas cells of the low line did not.

In irradiated mice, the latent periods for development of tumors from cells of the two lines differed significantly. This difference between the two cell lines could result from different rates of cell proliferation or cell necrosis *in vivo*. The object of the third study was to determine whether cells of the high line did proliferate more rapidly than low-line cells when first transferred to the mouse, and, if so, whether this higher rate of proliferation was an inherent property of the high-line cells characterizing their growth *in vitro* as well as *in vivo*. The study revealed that cells of the high line proliferated and grew more rapidly than low-line cells when first implanted into mice but that cells of the two lines proliferated at approximately equal rates *in vitro* under the conditions of these experiments.

Materials and Methods

The source of cells, culture procedures, method of intramuscular injection of the cells, and the X-irradiation procedure for the mice were described in a previous paper (*3*). The culture fluid used throughout these studies consisted of 40 percent horse serum, 20 percent filtered chick-embryo extract (*7*), and 40 percent Earle's saline.

The following method was used for the immunization experiments of the first study: Strain C3H/HeN mice (1 to 6 months of age) were injected in the tip of the tail with 0.1 ml. of a concentrated cell suspension of the high-line cells or intramuscularly in the foreleg with 1 ml. of a suspension of the other types of cells tested (approximately 7×10^6 cells per mouse). The cells were suspended in culture fluid. Two weeks after the first injection, both the experimental mice and the noninjected control mice of the same age were challenged by injecting 1 ml. of cell suspension into the thigh muscles of the left leg. Cells were pooled from several T-60 flask cultures and approximately 4 to 8×10^6 cells were injected into each mouse. Both the experimental and control mice challenged with cells of the low line (and also cells of strain L (*8*, *9*) used in some of

these experiments) were X-irradiated[4] with a whole-body dose of 425 r approximately 6 hours before challenge because these cells fail to grow progressively in nonirradiated mice of strain C3H. The mice challenged with cells of the high line were not irradiated, except as indicated. After injection, all mice were examined at weekly intervals.

The mice used for assay in the second study were males of strain C3H/HeN, 1 to 2 months old. The irradiated mice received a whole-body dose of 425 r[5] 3 to 7 hours before injection of the cells. Sieved suspensions (*10*) of the cells in 1 ml. of culture fluid were injected into the muscles of the left thigh. The numbers of cell nuclei in aliquots of the suspension were determined by the procedure for enumeration of cell nuclei (*11*). After injection, the mice were examined weekly for 3 or 4 months. Autopsies were performed on all mice, and diagnosis of sarcoma was made by microscopic examination of tissue sections fixed in Zenker-formalin and stained with hematoxylin and eosin or with van Gieson's picrofuchsin.

For some observations on the growth and vascularization of the cells *in vivo*, the cells of each line were injected as a suspension in 0.2 to 1 ml. of culture fluid into the thigh muscles of both nonirradiated and X-irradiated mice. The number of cells injected was estimated by enumerating the cell nuclei in an aliquot of the suspension (*11*). At daily intervals after injection, mice were killed; 3 to 6 strips of tissue at the injection site were removed from each mouse, fixed, sectioned, and stained as described above. These sections were studied with particular attention to the mass of tumor cells, degree of necrosis of the injected cells, cellular infiltration of muscle, vascularization of the tumor-cell mass, and cellular reaction of the host. The sections were also examined at a high magnification (× 1,455) to determine the percentage of cells in mitosis. Cells were counted in the center of 10 to 20 randomly selected fields of 3 to 6 sections of tissue from each mouse, and the number of cells in mitosis (excluding the earliest stages of prophase) was determined. For each mouse, between 300 to 2,000 cells were enumerated.

Cells of each line were also implanted in transparent chambers in the mouse according to the techniques developed originally by Algire and Legallais (*12*) and modified by Merwin and Hill (*13*). A concentrated suspension of the cells in culture fluid was injected by capillary pipette into a pocket made in the subcutaneous tissues. The growth and vascularization of the implant were studied by microscopic observations in the living animals and by serial sections of fixed and stained preparations.

The test for acquired immunity to homologous cells was that developed by Merwin and Hill (*13*). In each test, harderian-gland tissue, taken from a strain C57BL/6JN mouse 1 to 3 days old, was placed in the subcutaneous tissues of the strain C3H mouse so that the graft could be observed microscopically through a transparent window. The graft was implanted so as not to become vascularized. Such nonvascularized grafts can serve

[4] The authors express their appreciation to Mr. Henry Meyer, of the Radiation Branch of this Institute, who irradiated the mice in all of these studies.

[5] The physical factors differed slightly from those used previously and were as follows: 200 kv., 15 ma., 120 r per minute at a focal distance of 54 cm. through 0.25 mm. Cu and 0.55 mm. Al filters.

as indicators of acquired immunity since they do not initiate immunity but disintegrate if immunity develops in the host to cells of the same strain as the graft. Some of the mice were X-irradiated with either a whole-body sublethal dose of 425 r or a lethal dose of 900 r. Each of the latter mice was protected by an intravenous injection of isologous (C3H) bone marrow, the marrow from one femur suspended in 0.5 ml. Tyrode's solution. Twelve hours after irradiation, each of the irradiated mice and nonirradiated controls of the same age were injected intramuscularly with a spleen (or ½ spleen) of a mouse of the same strain as the harderian gland (C57BL/6JN). The spleen cells were suspended in 1 ml. of the same type culture fluid used for the tissue-culture cells. At almost daily intervals after injection, the harderian-gland implants were examined microscopically for disintegration of the pigment cells, which indicated the development of immunity to the homologous spleen cells.

In order to ascertain the rate of cell proliferation *in vitro*, replicate cultures were prepared from sieved and stirred cell suspensions according to methods previously described (*10*). At the time of inoculation, and after growth for 1 or 2 weeks, cultures were selected at random for the enumeration of cell nuclei (*11*).

Experimental

Study I: Capacity of Cells to Induce Immunity in Mice of the Strain of Origin

The object of the first group of experiments was to determine whether cells of the low and high sarcoma-producing lines induced immunity in mice of strain C3H and whether the two lines cross-immunized mice of this strain. As may be noted (table 1) cells of the low line induced immunity in the mice that completely prevented the continued growth

TABLE 1.—*Results of experiments testing whether cells of the low sarcoma-producing line immunize mice of strain C3H against cells of either the low or high sarcoma-producing lines*

Months *in vitro* of low-line cells used for first injection	Results of challenge: Mice with sarcomas* / Number of mice injected			
	Low line in X-irradiated hosts		High line in nonirradiated hosts	
	Experimentals	Controls	Experimentals	Controls
36	0/20†	9/19	—	—
38	0/14	2/14	—	—
39	0/17†	11/20	—	—
34	—	—	1/8‡	6/6‡
36	—	—	10/17	5/5
41	—	—	9/15	14/15

*In this and the following tables the denominator represents the number of mice injected and surviving; the numerator represents the number that developed sarcomas at the site of challenge.

†One mouse developed a sarcoma at the site of first injection in each of these experiments, but the cells at the site of challenge did not produce a tumor.

‡Results at 11 days after challenge. By 3 months after challenge 6 additional sarcomas had developed in the experimental mice.

of the low-line cells and delayed growth of the high-line cells used for challenge. Cells of the high line (table 2) similarly induced immunity in the host; this immunity, with one exception, completely prevented the continued growth of cells of either line used for challenge. It was thus shown that cells of each line induced immunity in the mice and that the two cell lines produced cross-immunity in mice of the strain of origin (C3H).

TABLE 2.—*Results of experiments testing whether cells of the high sarcoma-producing line immunize mice of strain C3H against cells of either the high or low sarcoma-producing lines*

Months *in vitro* of high-line cells used for first injection	Results of challenge: Mice with sarcomas / Number of mice injected			
	High line in nonirradiated hosts		Low line in X-irradiated hosts	
	Experimentals	Controls	Experimentals	Controls
49	0/4	2/4	—	—
50	1/5	4/5	—	—
51	0/4	4/5	—	—
51	0/4*	4/4*	—	—
48	—	—	0/4†	4/5
50	—	—	0/5	4/4
51	—	—	0/4	6/6

*The mice in this experiment were X-irradiated before challenge.

†One mouse developed a sarcoma at the site of first injection, but the cells at the site of challenge did not produce a tumor.

In view of these findings, it seemed of interest to determine whether cross-immunity might be demonstrated between cells of the two lines and cells of a clone of strain L (*8*, *9*) that originated in culture 10 years earlier from another strain C3H mouse. Cells of this clone were found in a previous study (*5*) to immunize mice of strain C3H.

As may be noted (table 3), in each experiment such cross-immunity was demonstrated. The first injection of cells of one clone reduced the number or retarded the development of sarcomas from cells of the other clone.

In determining whether the tissue-culture cells immunized the host, the cells were injected as a suspension in a culture fluid of dilute horse serum and chick-embryo extract. Washed cells were not used since it is doubtful whether saline washings would rid the cells of all foreign proteins; furthermore, repeated washings with balanced saline were found to damage the cells. It was thus necessary to establish whether the immunity was induced in the mice specifically by the injected cells or was in some way associated with the heterologous culture fluid.

Tests were made (table 4) to determine whether injections of culture fluid, either alone or as a suspending agent for living cells, increased the resistance in mice to the subsequent growth of cells of the two clones studied. There was no indication from these experiments, either in the incidence of tumors produced or in the period required for the develop-

TABLE 3.—*Experiments testing for cross-immunity between cells of the low or high sarcoma-producing lines and cells of a clone of strain L*

Cells used for first injection	Months *in vitro* of cells used for first injection	Results of challenge: Mice with sarcomas / Number of mice injected			
		Low or high line		Clone of strain L	
		Experimentals	Controls	Experimentals	Controls
Clone of strain L	162	9/14*	14/15*	—	—
	164	1/6	5/6	—	—
	165	2/5	8/8	—	—
	166	1/2	2/3	—	—
	168	3/7	6/7	—	—
Low line	42	—	—	4/14	12/15
	47	—	—	3/7†	6/7†
	48	—	—	2/8‡	6/10‡

*The mice in this experiment were challenged with cells of the high line; the mice in the following 4 experiments were X-irradiated and challenged with cells of the low line.

†Results at 54 days after challenge; by 90 days 3 more sarcomas had developed in the experimental mice.

‡Results at 33 days after challenge; by 90 days all experimental and control mice had developed sarcomas.

ment of the tumors, that the injected culture fluid in the absence or presence of living cells induced immunity in the mice effective against cells of either of the two clones. Although the culture fluid may have served as an adjuvant, the immunity induced in the mice must have developed specifically from the injected cells.

TABLE 4.—*Experiments testing whether culture fluid in the presence or absence of living cells increases resistance of mice to cells of the two lines or the clone of strain L*

Materials used for first injection	Experiment number	Results of challenge: Mice with sarcomas / Number of mice injected			
		Clone of strain L		Low or high lines	
		Experimentals	Controls	Experimentals	Controls
Culture fluid	1	7/7	7/7	—	—
	2	—	—	13/17	5/5
	3	—	—	5/6*	6/7*
Culture fluid and homologous tissue cells†	4	4/4	5/5	—	—
	5	2/2	4/5	—	—
	6	—	—	14/15	14/15
Culture fluid and heterologous tissue cells‡	7	7/8	7/7	—	—
	8	5/5	5/5	—	—
	9	5/5	4/5	—	—

*The mice of this experiment were X-irradiated and challenged with cells of the low line; in the other two experiments the mice were challenged with cells of the high line.

†The homologous tissue cells injected with the culture fluid consisted of skeletal muscle of mice of strain C57BL/6JN (expts. 4 and 5) and of strain A/LN (expt. 6).

‡The heterologous tissue cells were from strain HeLa (*14*) that had been cultured for the previous 5 months in a culture fluid of horse serum, chick-embryo extract, and saline (*15*).

Since cells of the two lines were antigenic to mice of strain C3H, it seemed possible that the cells might grow in mice of some foreign strain. Therefore, approximately 20 mice of each of 9 inbred strains (A/L, A/He, DBA/2, BALB/c, C57BR, C3H/He, C3Hf/He, C57BL, and C57L)[6] were inoculated with cells of the high line or with sarcomas developed from cells of the high or low sarcoma-producing lines. Cells of the low line previously were found (*3*) to produce sarcomas in nonirradiated mice of strain C3H after one passage in X-irradiated hosts.

The cells of the two lines grew in all mice of strain C3H but failed to grow in any mice of foreign strain, with one exception; this occurred in a mouse of strain DBA/2 that developed a sarcoma from the high line. This sarcoma was successfully transplanted to 1 of 5 strain DBA/2 mice subinoculated.

Study II: Relationship Between Tumor Latent Periods and Development of Immunity in the Host

Tumor latent periods[7] *and incidence of sarcomas in irradiated and nonirradiated mice.*—Cells of the two lines were compared with respect to the periods required for the development of palpable tumors from known numbers of cells. The results of these studies may be summarized as follows:[8] In nonirradiated mice (text-fig. 1) the tumor latent periods for the high-line cells tended to vary inversely with inoculum size. No regression of tumors was observed in these studies. Only 3 mice developed tumors from the low-line cells, and the latent periods for these tumors were significantly longer than those obtained from injecting equal numbers of high-line cells.

The size of inoculum in numbers of cells injected directly influenced the percentage of mice developing sarcomas from cells of either line. Large numbers of cells were necessary to produce tumors. Even with the high sarcoma-producing line, a mean inoculum of five million cells produced sarcomas in only 50 percent of the mice injected. A comparison of these data with those of the earlier assays (*3*) indicated that the sarcoma-producing capacity of the high-line cells as measured by the frequency with which the cells produced sarcomas had decreased during the years of growth *in vitro*, whereas that of the low-line cells had remained almost constant.

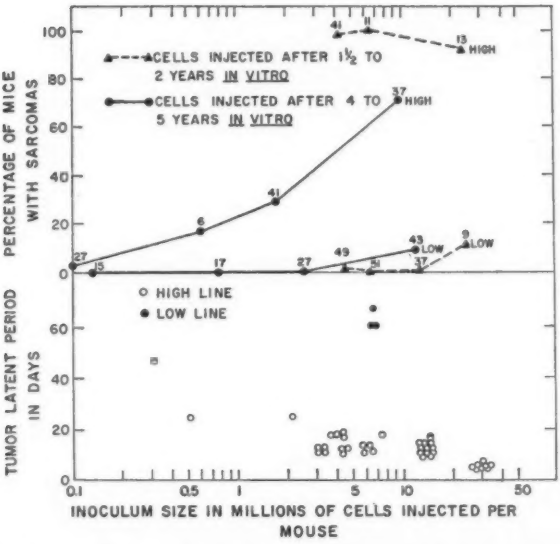
In X-irradiated mice (text-fig. 2) the tumor latent periods tended to vary inversely with inoculum size and at each inoculum level were significantly longer for cells of the low line than for cells of the high. The tumors produced by the low-line cells also grew more slowly than those produced by high-line cells.

Irradiation of the host significantly increased the percentage of mice

[6] The authors express their appreciation to Dr. Lloyd Law, of this Institute, who helped with this phase of the study and supplied mice of the inbred strains used.

[7] The "tumor latent period" measured the interval from the injection of cells to the appearance at the injection site of a palpable tumor that continued to grow progressively as a diagnosed sarcoma.

[8] The authors express their appreciation to Mr. Nathan Mantel, of the Biometry Branch of this Institute, for his helpful suggestions in the presentation and analysis of data in this study.

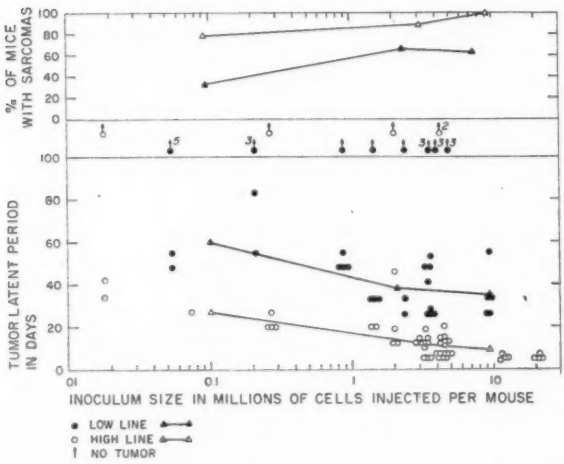

PERCENTAGE OF MICE WITH SARCOMAS
CELLS INJECTED AFTER 1½ TO 2 YEARS IN VITRO
CELLS INJECTED AFTER 4 TO 5 YEARS IN VITRO
HIGH
LOW
TUMOR LATENT PERIOD IN DAYS
HIGH LINE
LOW LINE
INOCULUM SIZE IN MILLIONS OF CELLS INJECTED PER MOUSE

TEXT-FIGURE 1.—Results of injecting cells of low and high sarcoma-producing lines into nonirradiated mice of strain C3H after the cells had been cultured for 4 to 5 years *in vitro*. Results of earlier assays (*3*) after 1½ to 2 years of growth *in vitro* are included[9] for comparison. In text-figure 2, the *lower panel* shows individual latent periods of tumors plotted against inoculum size. *Top panel* shows lines describing the percentage of mice developing sarcomas as functions of inoculum size. For each of 4 inoculum-size groups, the percentage of mice developing sarcomas was plotted against the geometric mean inoculum received by mice in the group. Number *above* each determination indicates number of mice injected.

developing sarcomas. For the production of sarcomas in 50 percent of the mice injected with cells of the high line, a mean inoculum of 5×10^6 cells was required in nonirradiated mice but considerably less than 10^5 cells in X-irradiated mice. Similarly, for production of sarcomas in 10 percent of the mice injected with cells of the low line, a mean inoculum of more than 10^7 cells was required in nonirradiated mice and considerably less than 10^5 cells in X-irradiated mice. The percentage of mice developing sarcomas from the low-line cells was slightly higher than that observed in the assays made after 1½ to 2 years of growth *in vitro* (*3*).

Period required for development of immunity to homologous cells.—By means of the harderian-gland test, the development of immunity to a massive injection of homologous spleen cells was measured in both X-irradiated and nonirradiated mice of strain C3H (table 5). The following results were obtained: In mice irradiated with a sublethal dose, immunity did not develop until an average of 15 days after injection of homologous cells. The use of a lethal dose of X irradiation followed by the injection

[9] In these earlier assays the cells from one T-60 flask culture (approximately 3×10^7 cells) were injected intramuscularly into 1 mouse or into groups of 2 to 6 mice of approximately the same age as those used in the present study. Although the number of cells injected into each mouse was not measured, the approximate number could be estimated from the preceding data. These calculated values are used in this chart.



TEXT-FIGURE 2.—Results of injecting cells of the low and high sarcoma-producing lines into X-irradiated mice of strain C3H after the cells had been cultured for 4 to 5 years *in vitro*. *Arrow* pointing upward (*top of lower panel*) indicates that no tumor appeared within the period of observation (90 days). If several mice failed to develop tumors, the number of these mice is indicated. *Lower panel* shows lines describing the average latent period for developed tumors as functions of inoculum size. For this purpose, mice developing tumors were divided into the same 3 inoculum-size groups used in *upper panel*, and the average latent period for each group was plotted against its geometric mean inoculum. All average values are indicated by triangles.

of isologous bone marrow did not lengthen the time for development of homologous immunity. In nonirradiated control mice, immunity developed in an average of 7 days after injection.

During the period in which there was no evidence of the development of immunity to homologous cells, the high-line cells produced palpable tumors that continued to grow progressively, whereas approximately equal numbers of low-line cells failed to produce any tumors during this interval, and sarcomas developed only after a prolonged latent period.

Study III: Growth of Cells in Vivo *and* in Vitro

Growth and vascularization of implants in strain C3H mice.—A study of the intramuscular and subcutaneous growth of the cells revealed the following differences between the two cell lines: As early as 1 to 3 days after injection into either irradiated or nonirradiated hosts, the percentage of cells in division was regularly threefold to fivefold higher among cells of the high line than among cells of the low (table 6). By 5 days after injection, the cells of the high line formed larger masses, were better vascularized, and infiltrated the muscle more extensively than did cells of the low line (figs. 1–6). Any host cellular reaction to the implants was negligible during the period of observation. There appeared to be no difference between the two lines in the degree or extent of cell necrosis

TABLE 5.—*Comparison of individual latent periods for development of immunity to homologous cells and for development of tumors from the low- and high-line cells in X-irradiated mice of strain C3H*

Cells injected	1			2		3	
	52–58-Day-old mice			29–45-Day-old mice		87–92-Day-old mice	
	No irradiation	425 r		425 r		900 r+bone marrow	
	Individual latent periods* (*days*)	No. cells injected ($\times 10^6$)	Individual latent periods* (*days*)	No. cells injected ($\times 10^6$)	Individual latent periods* (*days*)	No. cells injected ($\times 10^6$)	Individual latent periods* (*days*)
Low line	—	9. 1	97 (3) Neg.	4. 1–5. 0	Neg. (6) d. (3)	5. 6	37 42 d. (6)
High line	—	7. 0	6 (4)	4. 2–4. 4	5 (3) 7 (5) 12 Neg. (2)	3. 6	7 (3) d. (5)
Homologous spleen	6 (3) 8	—	13 (2)† 17 (3) 21	—	10, 14‡ d. (4)	—	14 (5)§ d. (2)

*Individual latent periods represent the time of appearance of palpable tumors for mice receiving tumor-cell inocula, and time of development of demonstrable immunity for mice receiving homologous spleen. Where several mice in a group have the same latent period, their number is indicated in parentheses following the latent period. Neg. indicates that mouse survived the period of observation (90 days) without any tumor appearing. The symbol d. indicates that mouse died during the period of observation while either still tumor-free or non-immune. In total, there were 14 such deaths among groups receiving tumor cells. In all cases these deaths occurred prior to the appearance of any tumors in the respective groups in which the mice belonged, and, consequently, they should not affect the interpretation of the tumor latent-period data.

†One additional mouse dropped from observation on day 10 due to defect in transparent chamber.

‡Four mice died prior to development of immunity. Days of death were 10, 12, 14, and 17.

§Two mice died on days 4 and 16 without having developed immunity.

TABLE 6.—*Percentage of cells dividing in intramuscular and subcutaneous implants of low- and high-line cells in strain C3H mice*

Injection site	Treatment of mouse	Days after cell injection	Low line			High line		
			No. mice injected	Range of inoculum size in numbers of cells injected ($\times 10^4$)	Average percentage of cells dividing	No. mice injected	Range of inoculum size in numbers of cells injected ($\times 10^4$)	Average percentage of cells dividing
Intramuscular	Irradiated (425 r)	1	4	3–4	1. 1	4	3	3. 7
		2	4	3–4	0. 9	4	3	5. 2
		3	5	3–4	0. 4	5	3–4	4. 9
		4	6	3–4	1. 1	6	2–3	3. 9
		5	5 4	3–4 9–19	0. 4 1. 6	6 5	3–4 7–21	4. 7 3. 9
		7	3	4	2. 3	3	4	3. 1
		9	2	4	1. 8	3	4	4. 6
		10	4	10	3. 1	4	9	4. 7
	None	3	2	15	0. 2	2	16	4. 8
		5	2	13	0. 6	2	10	3. 1
		7	1	13	0	1	10	3. 7
Subcutaneous	Irradiated (425 r)	3	1	<0. 1	1. 2	1	<0. 1	4. 7
	None	3	2	<0. 1	0. 5	2	<0. 1	3. 7
		3	1	0. 1	0. 7	1	0. 2	3. 7

observed. By 10 days after injection into irradiated mice, implants of the low line showed a higher percentage of cells in division than observed previously (table 6).

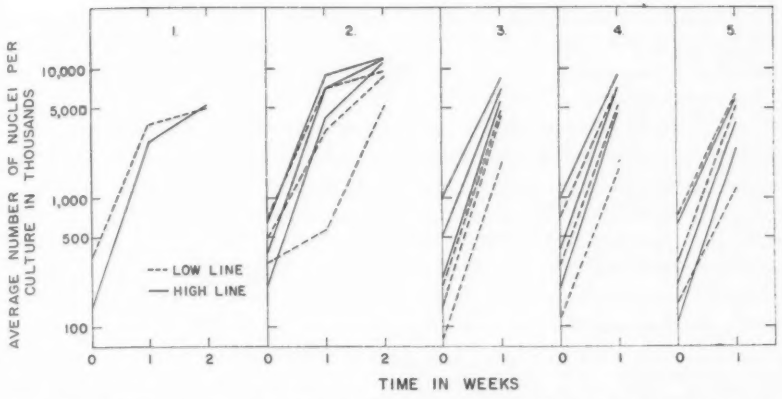
A difference in the host vascular response to cells of the two lines was observed in the transparent chambers. All implants of the high-line cells caused the nearby host vessels to become enlarged and convoluted within 3 to 4 days (figs. 7 and 8), whereas implants of the low-line cells caused no such reaction. These conclusions are based on an examination of 32 mice, 16 implanted with cells of each line; 3 sizes of inocula were used containing the following numbers of cells ($\times 10^5$): 0.2 to 4, 1 to 6, and 13 to 19; 2 of the 16 inocula were placed in irradiated animals and 2 in homologous hosts.

Nine implants from each line including two in irradiated hosts were observed in the transparent chambers for several weeks. The implants of low-line cells gradually disappeared in 10 to 30 days. The smaller implants including the two in irradiated hosts disappeared first. Some implants of low-line cells were penetrated by a few vessels, but this seemed to have no effect on their fate. Implants of high-line cells by the third or fourth day showed changes in opacity especially at the margin, probably indicative of growth. These implants were penetrated at the edge by a rich fringe of host vessels. This penetration began at about 4 days in those areas where the implant was close to host vessels. The cells grew so rapidly that they produced an opaque mass before the vessels could penetrate to the center of the implant. Later, most of the tumors of high-line cells became vascularized throughout. The small inocula did not produce palpable tumors, whereas the medium and large ones formed tumors 3 to 5 mm. in diameter. Regression, either a decrease in size or a cessation of growth, was evident by 11 to 25 days in non-irradiated mice. In the two irradiated hosts, the small implants of high-line cells were still growing after 30 days, at which time one had a diameter of 6 mm. and the other a diameter of 10 mm.

Proliferation of the cells in vitro.—The cells of the two lines were found to have approximately equal rates of proliferation *in vitro* (text-fig. 3). Although in two experiments (2 and 5) certain cultures of the low line exhibited a slightly lowered rate, this difference between the two lines was not consistent.

Discussion

The cells of each line were found to immunize mice of the inbred strain from which the cells originated. This finding indicated that some change, presumably of a genetic nature, had occurred either in the cells in the strain of mice or in both during the period of culture *in vitro*. According to the genetic laws of transplantation (*16*), tissue taken from an animal of an inbred strain should survive or grow in all animals of the same inbred strain unless genetic changes have occurred in the grafted tissue or in the host; for example, if the transplanted cells carry certain dominant genes not present in the host, the host usually develops immunity to the implant.



TEXT-FIGURE 3.—Experiments comparing proliferation of cells of the low and high sarcoma-producing lines *in vitro*. The average number of cell nuclei per culture was plotted on semilogarithmic grid against time in weeks. Each determination represents the average of 3 to 5 cultures (with the exception of one determination in experiment 1 in which two cultures were used). Since cell necrosis in these cultures was negligible, the rate of increase in numbers of nuclei approximated the proliferation rate of the cells. Cells used for these experiments had been cultured *in vitro* for 23 months (expt. 1), 52 months (expts. 2–4), and 55 months (expt. 5).

An observation reported in the first study gives some information on the possible origin and nature of the immunologic incompatibility between the two cell lines and the mice of strain C3H. Cells taken at different times from different mice of strain C3H were found after growth in culture to produce cross-immunity in mice of this strain. Furthermore, this immunity was shown to be induced specifically by the cells and not by the heterologous culture fluid in which the cells were growing. If the immunity observed was a typical antigen-antibody type of reaction, the cells must have had some antigen(s) in common that immunized the mice in which the cells were tested. In view of this finding, if the change(s) occurred in the strain of mice, the mouse used in 1940 to furnish tissue for strain L must have had a genetic constitution like that used in 1950 to supply tissue for the two cell lines, but these two mice must have differed from the mice used 3 years later in which all the tissue-culture cells induced immunity.

It seems more reasonable to assume that the change(s) occurred in the tissue-culture cells. However, if this is true, both of the lines and clones of cells tested underwent a similar change. Whether this antigenic change can be associated with any of the chromosomal changes that occur in cell populations during culture *in vitro* (*17*, *18*) or whether the antigenic change can be related to the malignant transformation in the cells awaits further study.

The results of the first study demonstrated that cells of both lines induced immunity in mice of strain C3H. However, the data do not exclude the possibility that the two lines might differ in the strength

of immunity induced; for example, the immunity acquired by the host during growth of the low-line cells might develop sooner and be more effective in preventing tumor growth than that induced by growth of the high-line cells.

The conclusion drawn from the second study is based on two assumptions: 1) that the harderian-gland test is a sufficiently sensitive indicator of the development of acquired homologous immunity; 2) that an intramuscular injection of the tissue-culture cells suspended in 1 ml. of culture fluid does not evoke a more rapid immune reaction in the host than an intramuscular injection of homologous spleen cells suspended in an equal volume of the same type of culture fluid. Several studies (*19–22*) have shown that the reaction of a host to a graft differs in intensity and rapidity in accordance with the degree of genetic relationship between the host and donor cells. Therefore, it would seem likely that the homologous spleen cells might evoke a more intense, rapid reaction in the host than the more closely related tissue-culture cells of isologous origin. If these assumptions are valid, the cells of the high line produced palpable tumors before immunity developed in the host, whereas an equal number of low-line cells did not; it may also be concluded that acquired immunity in the host is not the primary factor differentially retarding growth of the low-line cells during the first few days after transfer to the mouse.

An influence of the time in culture on the capacity of the cells to grow as sarcomas on implantation was observed. The high-line cells showed a decline in sarcoma-producing capacity after 3 years of growth *in vitro*. A similar decline was observed by Earle, Shelton, and Schilling (*23*) in six strains of C3H mouse cells cultured for 5 years and assayed in mice at yearly intervals. With the passage of time *in vitro*, each of these six strains lessened progressively in sarcoma-producing capacity. In both of these studies, the cells were cultured in a heterologous fluid of horse serum, chick-embryo extract, and balanced saline. In the present study, the cells of the high line exhibited the original high tumor-producing capacity when the cells were assayed in mice irradiated to impair the development of immunity; therefore, probably the decline resulted from an increased incompatibility between the tissue-culture cells and the host. Cells of the low line did not show a similar decline, and the injections into X-irradiated hosts revealed a slight increase in sarcoma-producing capacity, possibly indicating that further alterations had occurred in cells of the low line.

At least two factors may explain the excessively large inoculum required to produce sarcomas from cells of each line as compared with the numbers of cells required for successful transfer of tumors of *in vivo* origin (*16*). The observed antigenicity of the cultured cells for the host was undoubtedly a factor because a larger percentage of mice that had been irradiated to lower host resistance developed sarcomas from significantly fewer cells. However, even in irradiated mice several thousand cells failed to produce tumors in all animals, though the cells injected were healthy as indicated by microscopic examinations and culturing of aliquots of the cell suspen-

sions. The failure may have resulted from the ineffectiveness of irradiation in permanently preventing the development of immunity in the host, or it may have resulted from the sudden transfer of tissue-culture cells to conditions *in vivo* from conditions *in vitro* to which the cells had become adapted during the years of growth in culture. At the present time, no conclusion can be drawn regarding the relative importance of these factors.

Two interpretations could explain the prolonged tumor latent period exhibited by the low-line cells as compared with the high-line cells. First, the low line might have contained a smaller percentage of tumor-producing cells. Each inoculum of low-line cells would, therefore, have required a longer period to grow into a tumor. This interpretation would also explain the influence of inoculum size on sarcoma production. However, later studies [10] have shown that clones derived from single cells of the low and high lines also exhibited the characteristic difference in tumor latent period. A second interpretation is that cells of the two lines when first transferred to the mouse differed in rates of proliferation, necrosis, or both.

From observations of the third study the shorter tumor latent period exhibited by the high-line cells as contrasted with that of the low-line cells appeared to result from a more rapid rate of proliferation and growth of these cells when first transferred to the mouse, though the cells of the two lines were found to proliferate at approximately equal rates when cultured *in vitro* under the conditions of these experiments. It appears unlikely that acquired immunity in the host could have accounted for the difference in cell proliferation occurring as early as 24 to 72 hours after injection of the cells into either X-irradiated or nonirradiated mice. Apparently some other factor in the host environment, possibly nutritional or hormonal, differentially inhibited growth of the low-line cells and allowed rapid proliferation of the high-line cells.

Cells of the high line initiated an enlargement and convolution of host vessels that was never observed with implants of the low line. A similar correlation between this type of vascular response and the capacity of the cells to grow as sarcomas after implantation was observed by Algire, Chalkley, and Earle (*24*) in a study of cultured strains of C3H mouse cells implanted into strain C3H mice. The cell strains that produced sarcomas in more than half the mice injected induced a strong vascular response, whereas those that produced sarcomas in 0 to 11 percent failed to elicit any reaction. From an experimental study of tissue grafts in mice, Coman and Sheldon (*25*) concluded that the vascular response in the host was due to the proliferation of cells in the grafted tissue. In the present study the response may have resulted from the rapid proliferation of the high-line cells. It is also possible that some product of their metabolism initiated the response since the metabolism of the high-line cells differed from that of the low.

Woods, Hunter, Hobby, and Burk [in an addendum to Warburg (*26*)] found that cells of the high line exhibited consistently higher rates of

[10] Manuscript in preparation.

anaerobic and aerobic glycolysis approximately three times the values for the low-line cells. The rate of anaerobic glycolysis of the high-line cells was considered maximal for all cancer cells previously reported, including ascites cells. The rates of oxygen consumption were in the converse order, smaller for the high line than the low. This metabolic difference between the two cell lines and the significant difference in their utilization of glucose (*27*) appeared not to influence their rate of proliferation *in vitro* under the conditions tested, but this metabolic difference may somehow be associated with the different growth response of the cells when returned to the mouse.

Summary and Conclusions

Studies were undertaken to explain the difference in sarcoma-producing capacity of two lines of mouse cells derived *in vitro* from one cell. Two possibilities were considered. The first was that cells of the low sarcoma-producing line might differ from those of the high in being antigenic to mice of the inbred strain (C3H) from which the single cell originated. Cells of both lines were found to be antigenic to strain C3H mice and to produce cross-immunity in mice of this strain.

The second possibility was that cells of the high sarcoma-producing line might differ from those of the low in producing an established tumor before immunity developed in the host. Cells of the high line did produce palpable tumors in X-irradiated hosts before the host could develop detectable homologous immunity, whereas cells of the low line did not.

A study of the growth of the cells during the first few days after implantation in irradiated and nonirradiated mice revealed that cells of the high sarcoma-producing line proliferated more rapidly than low-line cells, induced an enlargement and convolution of nearby host blood vessels never observed with implants of low-line cells, formed larger masses, infiltrated the host tissues more extensively, and became better vascularized than equal numbers of low-line cells; during this period there appeared to be no difference between the two lines in the extent of cell necrosis *in vivo*. When grown *in vitro* under the culture conditions of these experiments, cells of the two lines proliferated at approximately the same rate.

It is concluded from these studies that the difference between the two lines in sarcoma-producing capacity does not result from the fact that one line is antigenic and the other not antigenic to the host since both were found to initiate immunity, but rather that the high-line cells produce an established tumor before resistance develops in the host. It is well known that such an established tumor can continue growth after immunity develops. The early production of an established tumor seems to depend on a more rapid rate of proliferation of the high-line cells *in vivo*, which is evidently due to a difference between the two lines in their response to some growth-controlling factor in the host.

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PLATE 11

FIGURE 1.—Representative area through growth of high-line cells 5 days after injection of cells (12×10^6) into the muscles of an irradiated mouse. *Note* large mass of tumor cells and infiltration of cells into the muscle. Van Gieson's picrofuchsin. × 105

FIGURE 2.—Representative area through growth of low-line cells 5 days after injection of cells (19×10^6) into the muscles of an irradiated mouse. *Note* smaller mass of tumor cells between the muscles and nerves and the less extensive cell infiltration into the muscle. Van Gieson's picrofuchsin. × 105

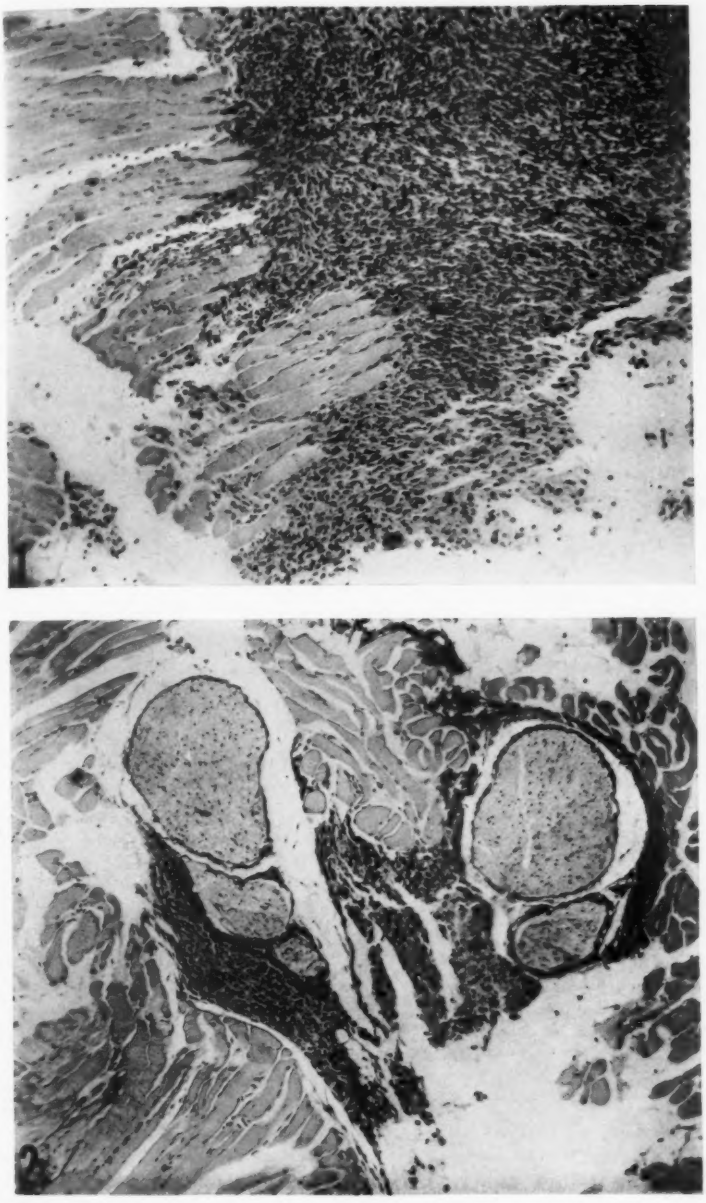

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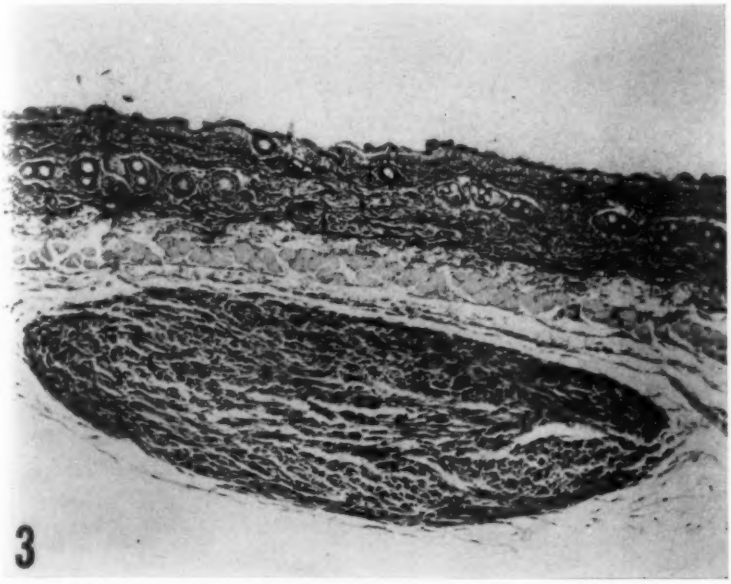
PLATE 12

FIGURE 3.—Representative area through the center of cell mass of high-line cells 3 days after injection of cells (4×10^4) into the subcutaneous tissues of a nonirradiated mouse. At this time there was a marked host vascular reaction. Van Gieson's picrofuchsin. × 105

FIGURE 4.—Enlarged area of figure 3 to show condition of cells. *Note* lack of a host cellular reaction at the periphery. × 515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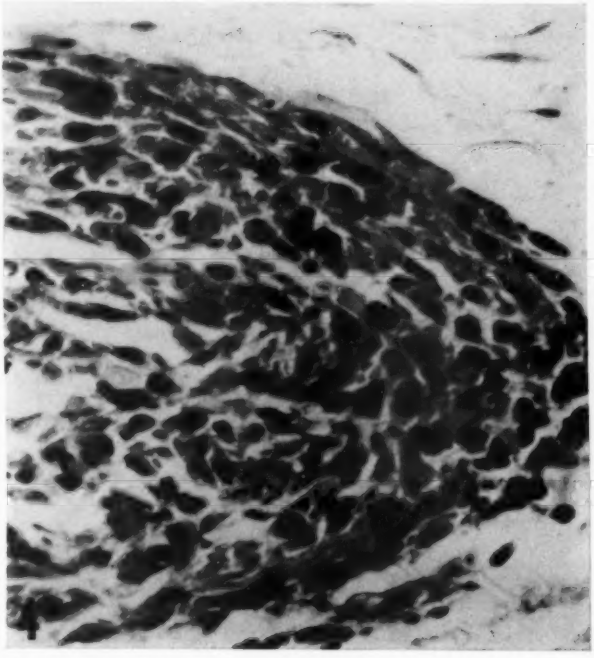

PLATE 13

FIGURE 5.—Representative area through the center of cell mass of low-line cells 3 days after injection of cells (4×10^6) into the subcutaneous tissues of a nonirradiated mouse. *Note* smaller mass of cell growth from the same size inoculum as that in figure 3. At this time there was no host vascular response; also the suspension of cells could not be seen in the living animal. Van Gieson's picrofuchsin. × 105

FIGURE 6.—Enlarged area of figure 5 to show condition of cells. *Note* lack of a host cellular reaction at the periphery. × 515

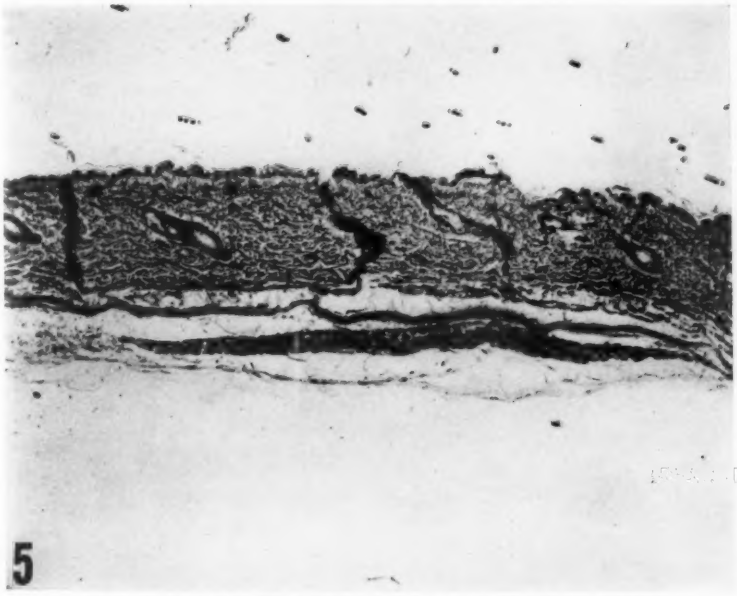
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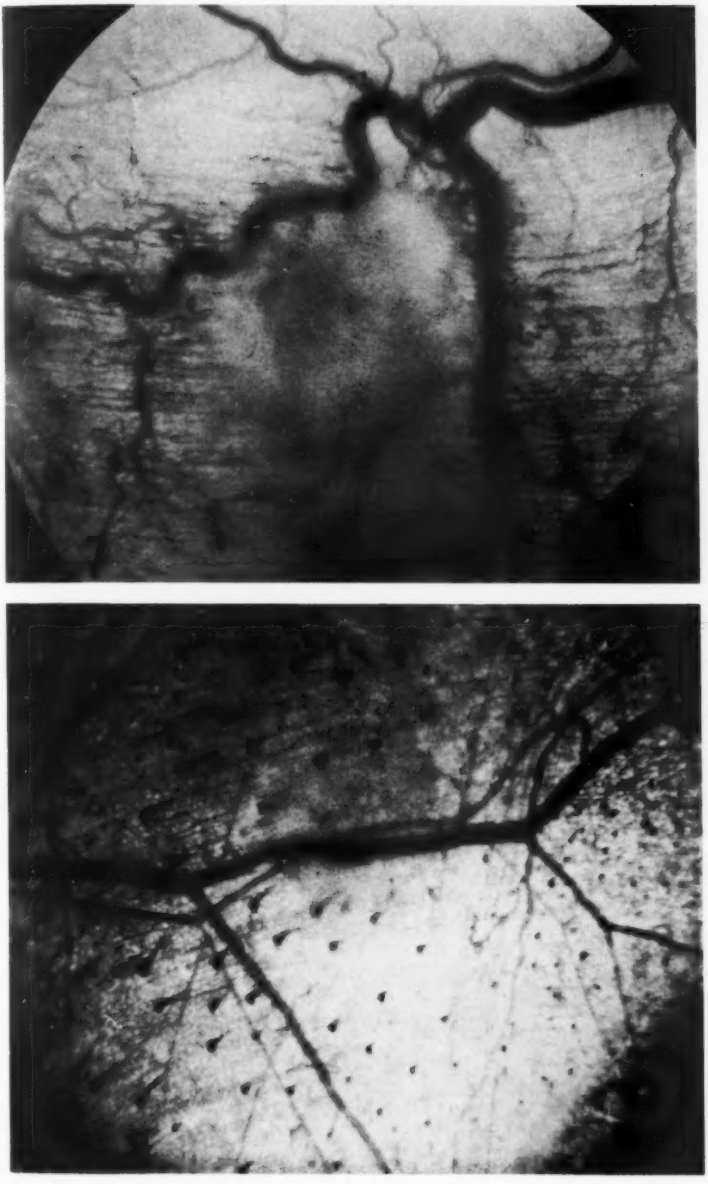
PLATE 14

FIGURE 7.—Host vascular reaction to cells of the high line 4 days after injection of cells (0.9×10^6). The cell suspension forms the central cloudy area. *Note* enlargement and convolution of the host vessels adjacent to the cell mass. × 24

FIGURE 8.—Host vascular reaction to cells of the low line 4 days after injection of cells (4×10^6). *Note* lack of host vascular reaction and the considerably smaller cloudy area of cells. × 24



Quantitative Studies on Rous Sarcoma Virus. I. The Titration of Rous Sarcoma Virus on the Chorio-allantoic Membrane of the Chick Embryo [1]

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The assay of Rous sarcoma virus (RSV) has received considerable attention in recent years. In large part this has been due to the widespread interest in the use of the Rous sarcoma as a convenient model for the detailed study of tumor virus: host cell relationships.

Keogh demonstrated in 1938 that the chorio-allantoic membrane (CAM) of the 12-day-old chick embryo would respond to the inoculation of RSV by the production of focal proliferative lesions, which he termed pocks (*1*). However, his report described the response of different eggs from a given lot as being too variable for reproducible virus assay by this method. Other workers since that time have had similar experiences (*2*). It is not surprising therefore that most workers continued to employ variations on the classical chicken assay. These assays underwent ultimate refinement by Bryan, who described experiments that were readily susceptible to rigorous statistical analysis, and in which a high degree of accuracy could be achieved (*3–7*).

Animal inoculation methods have the major disadvantage of requiring the maintenance and observation of a large number of animals for relatively long periods of time. The assays are therefore costly and slow, and require a great deal of space. For these reasons it was decided to reinvestigate the method of Keogh in order to determine whether the variability he found could be circumvented.

After the studies reported in this paper were under way, Rubin reported the first studies since 1938 in which Keogh's method was used (*8*). It was evident from his paper that Rubin did not encounter the variability that had led Keogh and others to abandon the method. The reason for this, which will become evident herein and in a following paper (*9*), lay in a fortunate choice of strain of chick embryo.

This paper will present studies bearing on the importance of strain of embryo for the assay method, and will present a detailed analysis of the

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447911—58——17

characteristics of the assay of RSV applicable to the most susceptible strain of embryo studied in our laboratory.

Materials and Methods

Virus strains.—The strain of virus used throughout the major portion of this investigation was derived from the standard Rous #1 chicken sarcoma, maintained in the laboratory of Dr. Francisco Duran-Reynals. Since 1955 this tumor has been maintained by weekly intramuscular transfer of minced tumor suspensions, through 1- to 3-week-old "Silver Cross" or "Pure Hope" White Leghorn chickens. The latter have been used exclusively during the past year because of more rapid and uniform response. Purified stocks of Rous sarcoma virus, supplied by Dr. W. R. Bryan of the National Cancer Institute, were used in some experiments.

Diluent.—All dilutions and extractions, unless otherwise specified, were made in a citrate solution slightly modified from that used by Bryan (*10*). The citrate buffer was prepared by a mixture of 1.0 *M* solutions of NaOH and citric acid in proportions to give a *p*H of 7.2. This stock solution was then diluted 1:20 for use. Immediately before use, the final solution was completed by the addition of 3 ml. of a stock solution containing 2 ml. inactivated horse serum, 5,000 units of penicillin, 5,000 μg. streptomycin, and 1 mg. of partially purified testicular hyaluronidase (supplied by the Wyeth Corporation, Philadelphia, Pennsylvania), which was frozen at −70° C., and stored at −35° C. in 3 ml. amounts.

Preparation of Virus Stocks

All stocks were initially derived from fresh 6- to 8-day-old tumor tissue, homogenized as a 10 percent suspension for 4 minutes at 0° C. in a Servel high-speed homogenizer. The resulting suspension was allowed to stand for 2 to 4 hours at room temperature, and then centrifuged at 4° C. for 30 minutes at 2,000 × g. The supernatant was termed the crude extract and was used in some experiments without further purification. In other experiments partially purified material was used which resulted from 3 alternate cycles of high- and low-speed centrifugation, with the above crude extract as starting material, and ending with a low-speed clarification. The above centrifugations were carried out in a Spinco Model L preparative ultracentrifuge, for 1 hour at 54,000 × g for the high-speed run and 10 minutes at 5,000 × g for clarification. All centrifugations were carried out at 0 to 2° C.

The final stocks were sealed in glass ampoules, frozen at −70° C., and stored in a dry-ice cabinet. Ampoules were thawed in a 37° C. water bath prior to use.

Chick Embryos

A variety of strains of chick embryos were utilized in these experiments. The majority were obtained from local breeders and will be designated by commercial strain designations. The White Leghorn eggs used in most of these experiments were obtained from a local source. The stock maintained by this breeder is renewed annually from the inbred

Pure Hope line maintained by Mt. Hope Farms, Williamstown, Massachusetts. Eggs for some experiments (White Leghorn, lines 9 and 15) were supplied by Dr. Burmester of the Regional Poultry Research Laboratory, East Lansing, Michigan. Fayoumi strain eggs were supplied by Dr. Arne Nordskog, Department of Poultry Husbandry, Iowa State College, Ames, Iowa. Duck eggs were donated by Carman River Duck Farm, Brookhaven, Long Island.

All eggs were received prior to incubation and were incubated at 37.5° C. in humidified incubators. Eggs were turned daily before inoculation.

Method of Inoculation

Eggs were candled after 12 days of incubation. Fertile eggs were selected whose chorio-allantoic membrane (CAM) extended more than half way around the shell. Two small holes were then drilled with a fine-grained carborundum disc attached to a small electric hand drill. One hole was located over the best vascularized portion of the CAM, usually adjacent to the largest blood vessel; the other was located at the apex of the blunt end, over the air sac. Great care was taken to drill the first hole without breaking or penetrating the shell membrane in order to minimize trauma to the underlying CAM. After drilling, a 1 cm. cuff of melted paraffin was applied around the lateral hole. The actual inoculation was begun within 1 hour of the time of drilling. An 0.1 ml. aliquot of material for inoculation was placed on the paraffined area of each egg to be inoculated. The inocula were then pulled onto the CAM by gentle separation of the fibers of the shell membrane with downward pressure by a stainless-steel dissecting needle, followed by gentle suction with a nipple at the air-sac end. No attempt was made at this time to drop the CAM completely, as this was accomplished under direct candling with mild houseline suction. The latter step was completed within 10 minutes of the time of inoculation. At the time of final candling, eggs with undropped membranes due to perforation and aspiration of inoculum into the allantoic fluid were discarded.

Harvesting and Counting Membranes

On the seventh day after inoculation, eggs were chilled overnight. The eggs were bisected for harvesting and the membrane from the upper half, containing the dropped area, was briefly washed in tap water. The membranes were then spread on glass plates, cut in 2 places with a razor blade, and completely flattened to avoid all folds. The membranes were counted by placing the plates on a black background and obliquely illuminating them with a fluorescent lamp placed approximately 2 inches above the level of the table. Pocks were enumerated with the aid of an automatic tally (Klett), and counted segments were marked with a water-soluble crayon to prevent overcounting.

General Titration Procedure

In most experiments 10 eggs were inoculated at each dilution with 0.1 ml. For reasons explained in this paper, geometric means of all

pock counts, other than 0, were then obtained. On the average, 8 countable membranes could be obtained when 10 were inoculated. The only membranes not used in calculation of data were those with no pocks or that died before the end of incubation.

Results

I. Effect of Strain of Embryo on Response to RSV

As indicated, the method of Keogh had been abandoned because of a prohibitive degree of egg-to-egg variability. In an effort to circumvent this difficulty, eggs of several strains were obtained from several breeders. These eggs were inoculated with aliquots of stable, partially purified, RSV stocks, prepared as outlined in the section on methods. In all except duck eggs characteristic focal lesions were noted at appropriate dilutions. Figure 1 shows a typical membrane inoculated with 300 pock-forming units. These lesions are small and can be clearly distinguished only with proper illumination on a dark background. With experience, they may be relatively easily distinguished from the nonspecific lesions which are seen in control membranes inoculated with saline. The latter type of lesions are usually larger and more irregular in shape. The possible error due to nonspecific lesions would be significant only if an attempt were made to derive data from membranes with an average of only a few pocks, since only a few nonspecific lesions are usually present per membrane.

The many strains examined differed primarily in two respects: 1) the number of eggs reacting with lesions after inoculation of potent virus inocula, and 2) the error variance of individual pock counts. These characteristics are summarized in table 1.

In all, the percent of nonreacting membranes is calculated from groups having an average pock count of greater than 20, thus rendering distributional zeros unlikely. In the local White Leghorn strain the percent

TABLE 1.—*RSV titration characteristics of different strains of embryo*

Strain	No. membranes tested	Nonreactors (%)	Error variance (*log*)	F*	*P*†
Duck	42	100.0	—	—	—
Fayoumi	48	75.0	0.39	5.58	<0.01
White Leghorn, line 9	44	55.2	0.33	4.71	<0.01
Cornish Rock	26	37.0	0.52	7.40	<0.01
Prod. barred	30	33.0	0.34	4.85	<0.01
Silver Cross	25	32.0	0.74	10.60	<0.01
Cornish Cross	24	25.0	0.18	2.56	<0.01
Sex-linked	66	19.0	0.11	1.57	<0.05 >0.01
White Leghorn, Pure Hope strain	300	11.4	0.07	—	—
White Leghorn, line 15	67	4.5	0.25	3.57	<0.01

*Variance test strain/variance Pure Hope strain. The degrees of freedom for the F test were derived from the number of reacting membranes in each group.

†Significance of difference between error variances of logarithms of individual pock counts compared with White Leghorn, Pure Hope strain.

of nonreactors was not correlated with dose in the range from 10 to 1,000 pock-forming units (p.f.u.) per membrane (p.f.u./0.1 ml. = geometric mean number pocks per membrane × dilution of inoculum). Insufficient data were available to determine whether this held true also in the other strains examined. It was of interest that estimates of titer of stable stocks were, within error, indistinguishable in all the lines examined, with the exception of the Fayoumi strain in which titers tended to be about tenfold lower. This comparison could not be made with assurance in the case of line 9 and 15, White Leghorn strains.

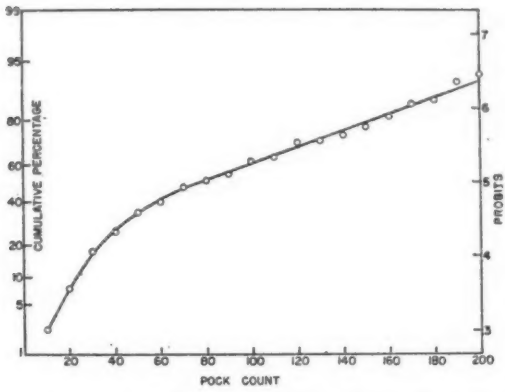
It was evident from these data that the local strain of White Leghorn embryos would provide the best material for further experiments aimed at providing a practical RSV assay. Studies relevant to the mechanism of the variability in this and the other strains will be presented elsewhere (*9*).

II. Characteristic of RSV Assay on "Pure Hope" White Leghorns

A. Distribution of individual pock counts.—In order to determine the best estimate of mean value in a series of membranes inoculated with aliquots of a virus suspension, it was necessary to determine the form of the distribution of individual pock counts. An experiment was therefore carried out in which 4 groups of 50 eggs were inoculated with 4 serial twofold dilutions of a virus stock whose estimated titer was 100 p.f.u./0.1 ml. inoculum. In order to avoid all bias, each egg was individually coded after inoculation, and then randomly distributed on new trays for incubation. The code was not broken until all counts were recorded.

To determine the distribution of individual pock counts, the data were referred to a single dilution. This was possible due to the essential linearity of the dose-response curve (estimates of titer at the four dilutions: 6.11, 6.21, 6.09, 6.14 log p.f.u./ml.). The Poisson distribution on theoretical grounds would be expected to fit the data if the only source of variation were sampling error. In such a distribution the variance is found to equal the mean. The variance of the distribution of pock counts was found in the present experiment to exceed the mean by factors of 25 to 50. It is thus clear that factors other than distributional error contribute major sources of variation. The distribution of individual pock counts, plotted on arithmetic probability (probit) paper, is presented in text-figure 1. If the distribution of individual pock counts is normal, such a plot will result in a linear curve. It will be seen that there is a marked departure from normality. It was thought that this curvature in the low pock-count range might be due to the practice of discarding all zeros as "nonreactors." However, replotting the data with all zeros included did not significantly affect the curvature. It was therefore concluded that an arithmetic mean would not serve as an unbiased estimate of central value. It should be noted that the bias introduced by simple averaging of data would be greatest in the preferred counting range of 10 to 60.

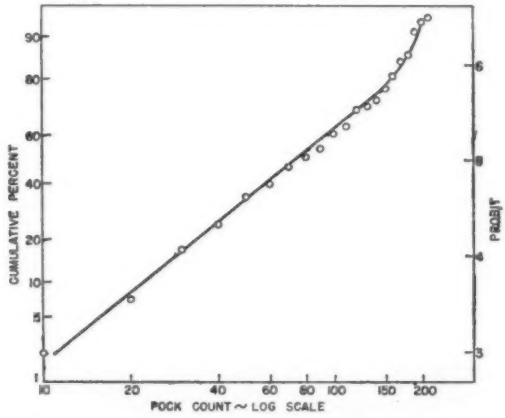
The effect of logarithmic transformation of the data may be seen in



TEXT-FIGURE 1.—Distribution of individual pock counts plotted on normal probability paper.

text-figure 2. It will be seen that the distribution is log-normal over the major portion of the countable range. The deviation at the upper end of the curve is not of sufficient magnitude to bias results seriously when geometric means are used in the usual countable range: mean pock counts of 5 to 150. This transformation was therefore adopted.

B. Accuracy of pock-count assay.—A major purpose of the individually coded experiments was the unbiased determination of error variance. As individual membranes vary somewhat in clarity and distribution of pocks, it seemed possible that pock counts on less clear membranes might be influenced by those obtained from more ideal membranes within a given group. Consciousness of this source of bias probably accounts for the fact that average error variance derived from 358 counts, obtained since the time of those given in table 1, was 0.11 as compared to 0.07 as

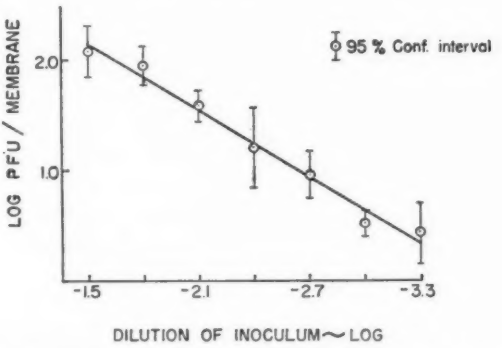


TEXT-FIGURE 2.—Distribution of logarithms of individual pock counts plotted on logarithmic probability paper.

previously found. The error variance of logarithms of individual counts in the coded experiment (137 counts) was 0.14. The difference between these variances is not of sufficient magnitude to warrant the expenditure of time involved in coding each inoculated egg.

Using the error variance obtained from the coded experiment, the confidence limits to be expected with any number of eggs may be easily determined. If 10 eggs are inoculated, and 8 membranes counted, the expected 95 percent confidence interval would be ± 0.31 log units, *i.e.*, a twofold error.[4] A similar degree of accuracy may be obtained with the latent-period chicken assay of Bryan by the use of at least 60 animals.

C. Relationship between dose and pock count.—That a linear relationship between dose and pock count exists was first described by Keogh (*1*) and confirmed by Rubin (*8*). It cannot be emphasized too strongly that this finding should be confirmed by any laboratory employing the pock-counting technique. Such linearity has not been established for all strains. The Silver Cross strain appeared, on the basis of limited data, to be very distinctly nonlinear, the curve appearing to fall rapidly at low dose levels. Furthermore, the finding of a linear relationship in a well-coded experiment confirms the ability to distinguish specific from non-specific lesions. Text-figure 3 summarizes an experiment in which serial dilutions were coded and then inoculated into 10 eggs per dilution. It will be seen that the relationship is linear over a range of 1.8 log units of concentration, *i.e.*, between an average of 3 and 140 pocks per membrane. It is of course preferable to avoid the extremes of this range, for reasons that have been pointed out. As has been clearly shown by Dulbecco (*12*), this relationship proves that each pock is the result of infection by a single "particle" not divisible on dilution. It should be emphasized



TEXT-FIGURE 3.—Relationship between dilution of inoculum and mean pock count.

[4] The standard error of the mean = $S_{\bar{x}} = \frac{\sqrt{s^2}}{\sqrt{n}}$

let $n = 8$, $s^2 = 0.14$

then $S_{\bar{x}} = \frac{\sqrt{0.14}}{\sqrt{8}} = 0.13$

The 95 percent confidence interval, using "Student's" t distribution may then be estimated: $t_{(0.05)}$ with seven degrees of freedom = 2.37, then the 95 percent confidence interval = $\bar{X} \pm 2.37 \times 0.13 = \bar{X} \pm 0.31$.

that this does not prove such a "particle" to be a virus particle (as opposed to a clump); nor does it indicate that every virus particle may infect and initiate a pock.

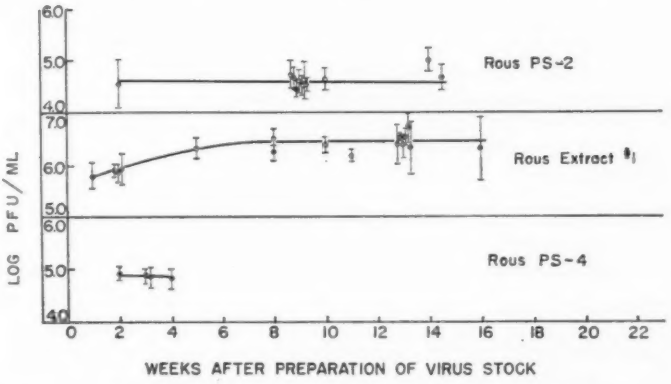
It will be noted that the confidence intervals do not vary significantly with dilution. That error variance on a log scale does not depend on mean pock count, within the range of 5 to 200 pocks, has been confirmed in many other experiments. This characteristic indicates that there is no advantage to be gained by designing these titrations with a large number of dilutions. For this reason it has been the practice to employ only a single dilution in assays where the end point can be predicted within a range of approximately ± 1 log.

D. Sensitivity of pock-count assay.—The absolute sensitivity of the assay, *i.e.*, the number of viable virus particles equivalent to a "pock-forming unit," cannot be determined unless particle suspensions of 100 percent viability can be counted by physical means. In the absence of such methodology it is possible only to compare sensitivity of one technique with that of another. In the case of the pock-counting technique this was done by Rubin, who compared his titers on standard stocks supplied by Dr. W. R. Bryan with those obtained by Dr. Bryan with the wing-web assay (*8*). Rubin reported pock titers to be essentially the same as the *average* of titers obtained by the wing-web method. Similar comparisons were carried out in this laboratory. Titrations were carried out on 6 coded virus stocks supplied by Dr. W. R. Bryan. In all cases, except for one stock that gave irregular results in Bryan's laboratory, *log-potency ratios* obtained by the CAM technique were not significantly different from those obtained by the wing-web inoculation method. However, the CAM technique, with the strain employed, appeared slightly less sensitive than the wing-web method when the most highly sensitive birds were used for the latter test. Bryan's stock CT581 was estimated to have a titer of 7.28 ± 0.25 log ED50 per ml. (*6*). This corresponds to an estimate of 7.12 "infectious particles" per ml. (ID50 × 0.693). The estimate derived from CAM titration was 6.66 ± 0.26 log p.f.u. per ml. On the other hand, when more resistant birds were employed the CAM assay was more sensitive, *e.g.*, Bryan's CT559 stock was estimated to contain 5.4 log ED50 units per 0.2 ml. inoculum (*13*), corresponding to an estimated 5.94 log "infectious particles" per ml.; which is significantly less than the estimate derived from CAM titration, which was 6.78 log p.f.u. per ml.

In order to determine whether the sensitivity of the CAM titration could be increased if virus were placed directly on the membrane, 5 experimental groups were titered on the same day by 2 methods. The first method was that described in the section on methods, the second consisted of dropping the membrane with 0.05 ml. saline in the usual way, and then introducing the virus with a #27 needle and syringe. It was found that the latter method was slightly more sensitive. The difference between the two being 0.30 log (± 0.28). Repetition of this comparison has always revealed differences between 0.1 and 0.3 log units. Such a

difference is to be expected since in the standard technique a proportion of the inoculum remains attached either to the outer surface of the shell or to the inner surface of the shell membrane.

E. Uniformity of response of different lots of eggs from the same strain.—In the majority of biological assays, there is a variation in sensitivity of the test material from week to week. If such a variability were present in the case of the CAM assay of RSV, it would be desirable to present all data in terms of log-potency ratios, relative to a stable standard stock, since the titer expressed in "pock-forming units" (p.f.u.) would not bear a constant relationship to the number of infectious virus particles. In order to investigate this point, successive assays on several stable stocks were analyzed. Text-figure 4 summarizes a series of such assays. In the case of PS-2 and PS-4 (semipurified stocks), no evidence of variation in susceptibility was noted. Rous extract #1 (a crude extract) appeared to increase in titer on storage at −70° C. This may represent variation in embryo susceptibility, or it may represent a dissociation of virus from inhibitor, or from clumps, after prolonged storage.



TEXT-FIGURE 4.—Estimates of titer of different Rous sarcoma virus preparations assayed at different times after preparation. PS-2 and PS-4 are semipurified preparations.

Since these assays were made, no evidence has accumulated in support of either of the preceding hypotheses because all titrations on stable stocks have yielded estimates of potency within a twofold range of variation about the mean. It is therefore likely that if heterogeneity in response exists among different lots of eggs, it must, at least in the case of the strain of eggs employed, be of small magnitude.

Discussion

The reason for the long neglect of the CAM titation for RSV becomes clear when the high degree of variability in response, which exists among the many available strains, is considered. It should be emphasized, therefore, that the near optimal titration characteristics described in this report

are applicable only to embryos derived from this and closely similar strains of chickens. With this reservation it may be seen that the CAM assay represents a practical means for the large-scale titration of RSV and Rous neutralizing antibodies.

The main drawback of the present method lies in the fact that 10 percent of embryos resist all doses of virus. That nonreacting embryos do not represent technical inoculation failures is evidenced by the constancy of this proportion despite increased technical proficiency attained in the course of this investigation; and this conclusion is further supported by genetic experiments which will be described elsewhere (*9*). The practice of discarding all zero counts has been shown by the data presented to result in no serious bias over the useful range of pock counts. Its main disadvantage lies in the possibility that a series of "nonreactor" zeros may, if the number of inoculated membranes is small, be interpreted as indicating an absence of virus. This error can effectively be eliminated only by use of a sufficient number of embryos to make such a distribution unlikely. If 5 membranes are counted, the probability of observing 5 zeros due to nonreacting membranes is $[(0.5)^5]$ 0.03, *i.e.*, once in 30 trials, on the average. If 8 membranes are counted, which is the practice in this laboratory, this probability is reduced to once in 300 trials.

It is expected that the problem of "nonreactors" may be eliminated by selective breeding experiments now in progress.

Summary

Evidence has been presented showing that different chicken strains vary widely in the proportion of embryos reacting to Rous sarcoma virus inoculated on the chorio-allantoic membrane, and in the uniformity of response exhibited by those embryos that do react. With one highly susceptible inbred line, it could be shown that virus assays with a high degree of accuracy could be obtained: 95 percent confidence intervals of $\pm$ 0.3 log requiring the inoculation of about 10 embryos. Pock counts were found to be log normally distributed, and their linear dependence on dose was confirmed. The sensitivity of this method of assay was found to be approximately equal to that of the wing-web route employed by Bryan.

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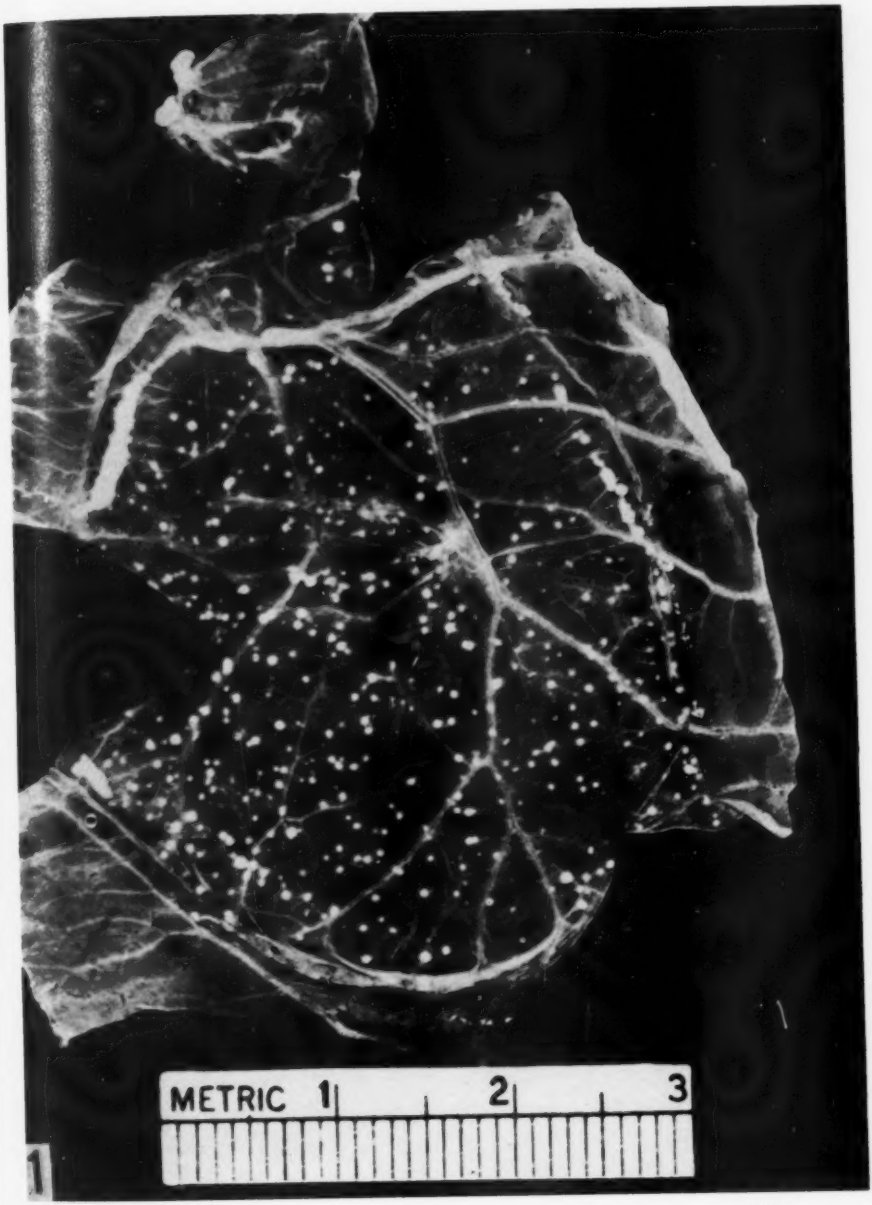
PLATE 15

FIGURE 1.—Appearance of membrane inoculated with approximately 300 pock-forming units of Rous sarcoma virus, 7 days previously.

Photograph by Dr. Fred W. Doyle.



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A Small-Animal Analytical Respirometer [1, 2]

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Studies of energy metabolism, through analysis of the respiratory exchanges, afford a measure by which the normal and the diseased physiology of the animal organism may be examined and compared. In recent years a variety of physical principles has been utilized for respiratory gas analysis (*1–6*). However, the progress in instrumentation for the study of respiratory gases has been channeled almost entirely toward the study of pulmonary physics in general (*7*). This communication describes new instrumentation of an analytical respirometer designed for continuous study of the expiratory exchanges of small experimental animals for any desired experimental period.

General Description of the Analytical Respirometer

The analytical respirometer consists of two parallel air lines supplied by a single source of dry gas. The air lines have been designated the reference air line and the test air line, for convenience. A schematic representation is given in text-figure 1. Reference gas in the form of compressed air is admitted at a common source, A, and is divided, through the interconnections, for flow into two air lines. The airstream in the reference line, after leaving the flow controller, D′, and the flow-rate meter, E′, passes sequentially through the reference side of the oxygen analyzer and the reference side of the carbon dioxide analyzer, and is then vented to atmosphere. The airstream in the test air line passes through the flow-controller, D, and flow-rate meter, E, and directly into the animal chamber, where it is partially depleted of its oxygen content and enriched with carbon dioxide and water vapor. The water vapor is removed in passage of the test airstream through a drier and the resulting dry gas introduced into the test side of the oxygen and carbon dioxide analyzers where it is compared with the reference gas. After passing through the analyzers, the test airstream is also vented to atmosphere. The use of

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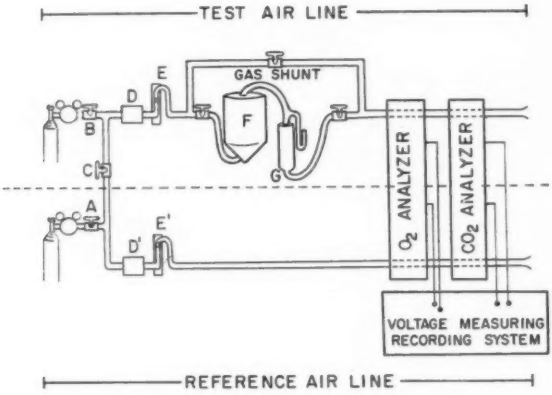
[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare



TEXT-FIGURE 1.—Schematic representation of analytical respirometer.

the reference gas in both air lines avoids significant variation in the concentration of argon and nitrogen in the two air lines. The analyzer chambers which contain the individual gas-sensing elements have been constructed as integral parts of the air lines. This arrangement presents the total volume of the respective airstreams to the sensing elements for analysis.

Commercially obtained tanks of dried, filtered, compressed air supply the reference gas used in this instrument. Gas from the tanks is introduced into the copper-tubing flow lines through a two-stage reducing valve. Equal flow of gas is maintained and monitored by inclusion of a flow controller [5] and flow-rate meter [6] in each air line. The flow controller operates by holding a constant pressure drop across an external needle valve. The differential applied to the valve is determined by a spring-loaded diaphragm which automatically controls a valve plunger and the flow through the needle valve. The companion flow-rate meters are variable-area glass tubes utilizing stainless-steel floats. The tubes are

TABLE 1.—*Relation of flow control to change in upstream pressure*

Pressures (*p.s.i.*)	Flow-rate meter reading	
	Reference (*mm.*)	Test (*mm.*)
4	73. 8	74. 0
6	73. 2	73. 0
8	73. 0	73. 0
10	73. 0	73. 0
12	73. 0	73. 0
14	72. 8	72. 5
16	72. 0	72. 0

[5] Constant Differential Flow Controller Model #62V. Moore Products Company, Philadelphia 2, Penna.
[6] Variable-Area Flow Rate Meter. Fischer and Porter Company, Hatboro, Penna.

graduated in millimeters over a length of 15 centimeters. Table 1 demonstrates the effectiveness of this system in maintaining a constant downstream flow over a limited range of variations in upstream pressure.

The test air line contains a gas shunt which allows the airstream to bypass the animal chamber, F, and drier, G (text-fig. 1). The shunt is essential to maintain continuous regulated flow of gas through the test side of the analyzers when the animal chamber is open. The shunt also allows the routine daily standardization procedures to be carried out simultaneously with the cleaning of chambers and handling of animals. For standardization of the instrument, individual standardizing gases are introduced into the test air line at B, and the needle valve C is closed, isolating the test air line from the reference gas source (text-fig. 1). The standardizing gases are synthetic air mixtures which have a reduced concentration of oxygen and an enriched carbon dioxide concentration in relation to the compressed air reference gas. The concentrations of argon and nitrogen are kept essentially the same as their respective concentrations in atmospheric air. The precise differential composition of the standardizing gases and the reference air gas is determined by mass spectrometric analysis.[7]

Metabolic chamber.—The important criteria governing the design of a metabolic chamber, satisfactory for long-term study of energy metabolism, are briefly summarized. The chamber must:

1) House the experimental animal for long periods of time—affording a living volume equal to the usual animal-room cage.

2) Maintain a constant ambient chamber temperature for the animal.

3) Provide food and water *ad libitum.*

4) Allow separate collection of urine and feces.

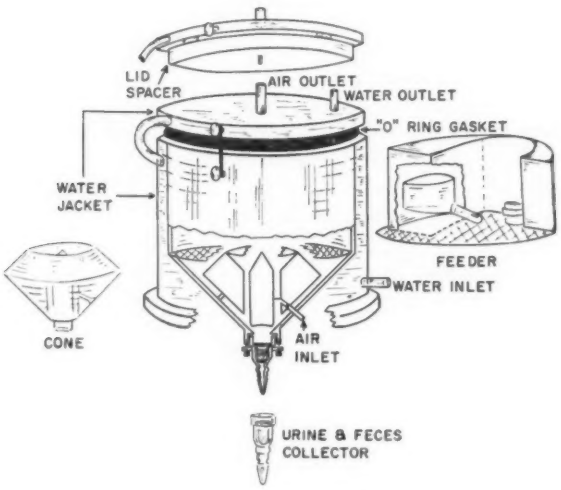
5) Allow continuous collection of the animal's total respiratory exchanges.

6) Be transparent to provide for observation of the animal at any time and, additionally, to avoid the hyperactivity usually associated with confinement of an experimental animal in a darkened cage.

The main body of the metabolic cage (29 cm. high and 24 cm. in diameter) has been constructed as a plexiglas cylinder (text-fig. 2). The advantages of plexiglas include transparency, durability, and the ease with which it may be worked. The surface is not easily wetted, which aids in collection of excreta. The low coefficient of heat exchange is of material advantage in maintaining a constant ambient temperature within the animal chamber. The base of the cage is a cone with a collecting funnel firmly seated at the narrow end, the stem of which has been shaped to fit the ground-glass joint of a graduated test tube. An outer plexiglas cylinder encloses the inner cylinder and most of the funnel-shaped base and forms a water jacket for maintaining a constant ambient temperature. A water-jacketed lid, of the same diameter as the outer cylinder, seals to the main body of the cage by pressure on a large "O" ring constructed from a piece of neoprene tubing. The snug seal of

[7] Model 21-103 180° Analytical Mass Spectrometer, Consolidated Engineering Corporation, Pasadena, Calif.

the lid above and the collecting funnel and test tube below give airtight junctions necessary in the measurement of respiratory exchanges. A lucite cone has been added in the base of the cage to reduce the cage volume and prevent direct excretion wastes into the collecting funnel. A wire-mesh floor of $\frac{1}{2}''$ hardware cloth rests on the cone and small supports that are cemented to the walls of the chamber.



TEXT-FIGURE 2.—The metabolic cage.

The excreta of the animal, after falling through the wire-mesh floor, are channeled to the collecting funnel. The collecting funnel contains a wire basket of small enough mesh to retain solid fecal material. The basket has been bent outward radially to form a supporting lip which, when seated circumferentially within the funnel, suspends the basket free of other contact with the funnel. When the excretions fall into the collecting funnel, they fall on the lip of the wire basket. The solid material goes into the basket and is retained while the urine passes through into the test tube without washing over the collected feces.

Food and water are available to the experimental animal at all times. A copper feeder, which fits snugly to the curve of the chamber wall, holds a food dish and a water bottle securely, to prevent spillage.

A light, plexiglas cage liner has been constructed as an aid in determining an accurate weight balance. The liner, which is the same shape as the inner surface of the cage, fits securely within the cage and contains the feeder, cone, and wire-mesh floor. The liner is easily removed from the cage for weighing.

In operation, compressed air enters at the base of the cage and flows upward over the animal and out through the annular opening between the chamber wall and the air space of the lid to the air-outlet tube. This design affords the longest mixing path for the respiratory air and has

been found to minimize transitory changes in gas composition that can be attributed to changes in the position of the animal's head with respect to the air outlet.

Gas analyzers.—Thermal conductivity is a physical characteristic of gases which offers unique possibilities for development of gas-analysis instruments requiring only simple electrical circuitry and a minimal amount of maintenance. The thermal conductivity method is based on the observation that when a wire, surrounded by a gas in a closed chamber, is connected to a constant-voltage source, the temperature of the wire changes until the continuous dissipation of thermal energy is equal to the electrical energy supplied the wire. The dissipation of thermal energy is accomplished by conduction, radiation, and thermal convection through the surrounding gas. By proper cell construction, radiation and convection, which account for a small portion of the dissipated heat, can be discounted entirely—making the equilibrium temperature of the wire solely dependent on the ability of the gas surrounding the wire to conduct heat. When the wire used has a high thermal coefficient of electrical resistance, this resistance will have a value corresponding to the thermal conductivity and, therefore, to the composition of the surrounding gas. By comparing the change in resistance of two such wires, one surrounded by a reference gas and the other surrounded by a test gas, the quantitative composition of the test gas may be readily and accurately determined if the qualitative composition of both gases is known. An accurate simple method of comparing two such resistances includes a Wheatstone bridge circuit.

Carbon dioxide analyzer.—A number of commercially obtainable carbon dioxide thermal conductivity cells have been found satisfactory for use in respiratory exchange analysis. The present equipment employs an Engelhard "high-speed, double bypass cell" that required only minor modifications for use in this application.[8]

Oxygen analyzer.—The oxygen analyzer is a Hays Corporation "Magno-Therm" unit that has been constructed as a differential analyzer for this specific application.[9]

The operation of the analyzer depends on the paramagnetic behavior of oxygen gas. A single cell block contains the reference-line and test-line sensing elements. The cell block is constructed of nonmagnetic metal with magnetic metal-pole pieces inserted in the opposite walls of both the reference cell and the test cell. A strong, permanent magnet bridges the two cells with its pole faces in line with the magnetic inserts. An electrically heated resistor in the form of a glass-covered filament of spiraled tungsten wire is mounted in the center of each of the cells with its lower end centered between the inserts. These heated resistors are connected with fixed resistors to form a Wheatstone bridge circuit.

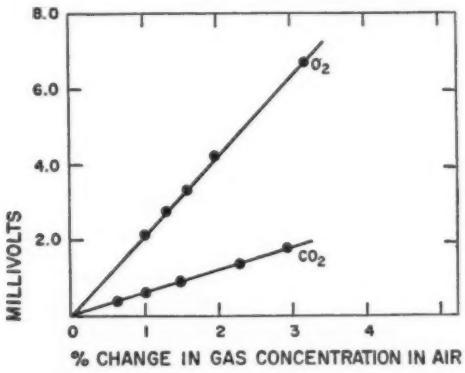
In operation, the oxygen-bearing gas is attracted into the strong magnetic field surrounding the heated resistor. The resistor is cooled as the surrounding gas molecules absorb heat. Hot oxygen gas loses its mag-

[8] Charles Engelhard, Inc., 850 Passaic Ave., East Newark, N. J.
[9] The Hays Corporation, Michigan City, Ind.

netism and thus is moved out of the magnetic field and upward along the resistor by the diffusion of cooler, more magnetic gas from below. Thus, a continuous circulation of gas is induced causing a flow-rate cooling effect which is in definite relation to the percent composition of the oxygen-containing gas entering the measuring cells.

The cells are housed in thermally insulated chambers and maintained in reference to room temperature in an air-conditioned room in which temperature fluctuations do not exceed 4° F.

Performance of the analyzers.—Over the range of values being reported, 0 to 3 percent depletion of oxygen in air and 0 to 3 percent enrichment of carbon dioxide in air, the output of the analyzers is a linear function of percent change in concentration of the respective gases in air (text-fig. 3).



TEXT-FIGURE 3.—Millivolts output per percent change of oxygen and carbon dioxide concentration in air.

The voltage outputs per percent change of the respective gases in air are given in table 2.

In the continuous, long-term study of experimental animals, it is necessary that the analyzers be electrically stable for 24-hour periods and, in addition, give reproducible zero and standardization values on a day-to-day basis. Zero output of the analyzers is achieved when reference gas is flowing in both air lines. The maximum variations in the daily zero and standardization values taken together over a 24-hour period is equal to an error of eight tenths of 1 percent (0.8%) in the CO_2 measurement and seven tenths of 1 percent (0.7%) in the oxygen measurement. Expressed as percent error of the measurement of 1 percent gas difference in air, the maximum error may be represented as 1.000 ± 0.008 percent for carbon dioxide and 1.000 ± 0.007 percent for oxygen.

The gas analyzers are sensitive to variations in pressure and it is essential that both air lines be maintained free of any pressure fluctuations. The gas measurements of the analyzers are on a partial-pressure basis; since the analyzers operate at atmospheric pressure with the flow rate accurately controlled and monitored and the air-line pressure main-

tained uniform, the analysis is on a true percent by volume basis. The carbon dioxide analyzer shows no change in voltage output per percent difference for flows up to 625 cc. per minute. The oxygen analyzer is insensitive to flow rates as high as 750 cc. per minute.

The relatively long life of the analyzers is apparent from the observation that the voltage output per percent change in gas concentration in air has remained essentially unchanged over the 2½-year period that the cells have been in use.

Power supply for bridge circuits of gas analyzers.—Power for the bridge circuits of the gas analyzers is supplied from two series-connected, 12-volt, wet cell storage batteries. The carbon dioxide analyzer is supplied with 135 ma. at approximately 2 volts. The oxygen analyzer is supplied with 98 ma. at approximately 22 volts.

TABLE 2.—*Instrument calibration values*

Oxygen		
Millivolts	% Gas	Mv./%
2. 13	1. 00	2. 13
2. 75	1. 28	2. 14
3. 37	1. 59	2. 12
4. 21	1. 98	2. 13
6. 75	3. 22	2. 10
		Average 2. 12

Carbon dioxide		
Millivolts	% Gas	Mv./%
0. 38	0. 63	0. 60
0. 62	1. 02	0. 61
0. 88	1. 47	0. 60
1. 38	2. 30	0. 60
1. 76	2. 94	0. 60
		Average 0. 60

Measuring and recording system.—The measuring-recording system currently in use in the instrument affords automatic digital-voltage measurement. It employs an analog to digital-voltage converter unit coupled with a circuit scanner unit, a programming unit, and an automatic electric typewriter.[10]

The analog to digital-voltage converter affords the measurement of analog functions appearing as low-level D.C. voltages. These voltages in turn are converted by the instrument into corresponding decimal numbers. The digital capacity of the instrument is 999 positive or neg-

[10] Telecomputing Corporation, Burbank, Calif. Teleducer—analog to digital-voltage converter. T. C. bulletin 104-1 Type 24A
Circuit scanner T. C. bulletin 112 Type 42A
Program unit T. C. bulletin 106 Type 33D
Electric typewriter T. C. bulletin 107

ative. In our application the full-scale voltage range is 0 to 10 mv. (0.000–0.010 v) with an accuracy of 0.1 percent of full scale.

Test voltages measured by the instrument are balanced against an internal reference voltage in a conventional Kelvin-Varley potentiometer circuit. The potentiometer is divided into discrete decimal increments. Balancing is accomplished by 3 unidirectional stepper switches which vary the reference voltage in increments of units, 10s and 100s; each of the 3 stepper switches operate sequentially and null detection is accomplished by a chopper amplifier.

The circuit scanner is a companion unit to the voltage converter unit, and its function is to connect sequentially a large number of data channels to the single voltage measuring unit. Controls are provided for manually selecting any given data channel for testing and monitoring purposes. In routine operation, the scanning rate for sequential reading of all data channels is fixed at 2-minute intervals and is under the control of an automatic cycling timer.

The program unit serves to interlock the circuits of the voltage measuring unit and the circuit scanner with those of the data-recording devices. The program unit also converts the digital presentation of the voltage converter into sequential form for recording. In addition, the unit affords the means whereby supplementary information such as automatic numbering of the readouts and identification of the input channel being measured and constants to be used in subsequent calculations may be introduced into the data records.

In this application, an automatic electric typewriter is used as the recorder device for visual display of the digital output of the voltage converter.

The use of multichannel equipment affords important economies in space and equipment plus the continuous monitoring of other important experimental parameters in addition to oxygen and carbon dioxide. The digital presentation of direct voltage measurements simplifies subsequent data handling, thus saving man hours.

Calibration of the instrument.—Calibration of the instrument to determine voltage output per percent change of the respective gases in air is done both at the beginning and at the end of each experimental run. Calibration is achieved by using an alcohol lamp and the mass spectrometer. The general procedure is to burn alcohol at a constant rate in the alcohol lamp, and to vary the amount of oxygen depletion and carbon dioxide enrichment by changing the rate of air flow through the combustion chamber. The lamp is constructed so that it may be directly substituted for the cage in the test line.

Standardization of voltage measuring system.—The zero balance, voltage span, and linearity of the voltage measuring equipment are monitored daily. Zero balance of the measuring bridge is determined with the terminals of an active input channel shorted together.

The voltage span and linearity of the instrument are standardized by introducing a series of known voltages into the instrument for measure-

ment. For this purpose a Leeds and Northrup type K potentiometer is used to obtain accurate voltage signals.

Standardization of the gas analyzers.—To insure proper function of the gas analyzers, the voltage output of the individual analyzer is measured when:

1) Reference gas is flowing in both the reference air line and the test air line.

2) Reference gas is flowing in the reference air line and a standardizing gas of known composition is flowing in the test air line.

Experimental Procedure

Data from all physical and chemical measurements are collected on a consecutive, daily basis. The experimental animal is maintained in the metabolic chamber for 23 hours out of every 24 hours.

Before beginning the daily experimental period, the following sequence of routine procedures is carried out to standardize the analytical respirometer:

1) The voltage measuring and recording components are standardized for zero balance and voltage range against an accurate, external voltage source.

2) With reference gas flowing in both air lines, the zero balance of the gas analyzers is determined for a 10-minute period.

3) Standardizing gas of known composition is introduced into the test air line and the voltage output of the analyzers determined against the known difference in oxygen and carbon dioxide concentration between the standardizing gas and the reference gas.

4) With standardizing gas still flowing in the test air line, the analyzers are switched to the recharged pair of batteries. After switching the batteries, the flow of standardizing gas is continued to determine that the voltage output per percent gas difference remains unchanged.

5) Following calibration with standardizing gas, reference gas is introduced into the test air line and the zero balance again obtained.

These simple procedures afford an accurate monitoring of the performance of the analytical respirometer. At the completion of the instrument standardization, reference gas is flowing in both air lines and the respirometer is again ready for use. Continuity of the test air line is maintained through the gas shunt during standardization. To begin the new observation period, the animal is returned to the metabolic chamber and the gas shunt closed.

While the instrument is being standardized, the animal is weighed, the food and water supply replenished, the cage washed, and the urinary and fecal wastes are collected. The air-line drier is recharged with fresh magnesium perchlorate adsorbent. All materials are weighed at the

beginning and at the end of the daily period and, where necessary, the volume noted. The difference in weight of the food cup and the water bottle is taken as a measure of food and water consumption. The urinary output is weighed and the volume measured. Fecal wastes are weighed in their wet state and the water content determined following drying of the feces for chemical analysis. The weight difference of the drier gives a measure of the water vapor lost by the animal during the daily period.

Results

It is apparent that the analytical respirometer is only a part of the total procedure concerned with the energy metabolism study of an experimental animal. To assess the accuracy of the complete procedure, including the analytical respirometer, a weight balance has been constructed for animals under study. By such a balance, the difference, expressed in grams, between the amount of substance taken in and the amount of substance excreted by the animal may be compared.

Calculation of a Weight Balance

Grams of gas.—The volume of oxygen consumed and carbon dioxide produced may be calculated by

$$V_T - V_Z = V \tag{1}$$

and

$$V\Big/\frac{\text{unit}}{\text{time}} \times F\Big/\frac{\text{unit}}{\text{time}} \times K = \text{Volume of gas in cc.}\Big/\frac{\text{unit}}{\text{time}} \text{ at STP} \tag{2}$$

where

V_T = total voltage output of the analyzer,
V_Z = voltage output of the analyzer at zero balance,
F = flow rate (volume of air in cc./min.) at STP, and
K = the calibration constant expressed as percent gas difference in air per millivolt output (%/mv.). (The calibration constant K includes the correction for the difference in affluent and effluent volumes when the R. Q. is not equal to 1.00, a correction based on the assumption that the nitrogen and other inert gases in air are only exchanged.)

Voltage measurements are obtained from the analyzer at 2-minute intervals. Thus, the values used in the calculation are the sums of the measured parameters taken for any desired number of consecutive 2-minute intervals.

The weight of the gas may be determined by multiplying the volume of gas by its density.

Animal intake in grams.—The weight of the intake of the animal is equal to the sum of the food eaten, the water taken in, and the oxygen consumed.

Animal output in grams.—The weights of the urinary and fecal wastes together with the carbon dioxide produced and water vapor lost to the drier give the total weight of the animal output.

TABLE 3.—*Daily weight balance*

Day	Grams in				Grams out					Weight difference
	Food	Water	O_2	Totals	CO_2	Drier	B.W.	Urine and feces	Totals	
1.....	13. 20	12. 08	13. 99	39. 27	16. 37	11. 70	7. 00	4. 01	39. 08	0. 19
2.....	9. 98	10. 31	13. 48	33. 77	15. 02	11. 80	2. 00	5. 59	34. 41	−0. 64
3.....	11. 70	13. 59	14. 00	39. 29	16. 37	12. 40	5. 90	4. 38	39. 05	0. 24
4.....	12. 11	11. 92	13. 25	37. 28	15. 75	12. 10	4. 20	5. 25	37. 30	−0. 02
5.....	11. 80	14. 57	14. 00	40. 37	16. 68	11. 60	6. 09	5. 90	40. 27	0. 10
6.....	12. 21	12. 14	13. 48	37. 83	16. 48	11. 20	5. 20	4. 99	37. 87	−0. 04
7.....	11. 90	11. 82	13. 18	36. 90	15. 54	11. 80	5. 00	4. 10	36. 44	0. 46
Weekly total.....				264. 71	.....				264. 42	0. 29

The 23-hour values for oxygen consumed, carbon dioxide produced, and water vapor lost to the drier are extrapolated to 24 hours. Whatever additional output of urine and feces occurs during the 24th hour is collected with the 23-hour specimens so that these wastes are collected over a 24-hour period. No food or water is available to the animal during the 24th hour.

The daily weight balance for a normal rat over a consecutive 7-day observation period is tabulated in table 3. The values recorded in the tables are the differences obtained in the weighings at the beginning and at the end of the daily observation period. It can be seen that the maximum error for any day is 1.8 percent (Day 2) when the weight difference is expressed as the percent error of the daily totals. It is felt that the weight difference is related primarily to the error in weighing the animal. It is apparent that the weight difference over the weekly period is small. The determination of weight balance in this manner is considered to be a satisfactory index of the degree of accuracy with which study of the total metabolism of rats may be done under these experimental conditions.

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Observations on the Energy Metabolism of Rats Receiving Walker Tumor 256 Transplants [1]

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There is extensive literature stressing the significant metabolic demands placed on the animal host by the presence of progressively growing tumor (*1–3*). In their review of the subject, Fenninger and Mider (*4*) have emphasized the need for intensive study of the energy metabolism to obtain better understanding of host-tumor relationships. With the completion of new instrumentation (*5*), it is now possible to study the metabolic performance of individual tumor-bearing animals over a prolonged experimental period. The data in this paper present some aspects of the alterations in energy metabolism observed in rat hosts receiving tumor transplants.

Materials and Methods

The experiments were done on young, male, Osborne-Mendel rats obtained from our own breeding colony.[3]

All animals weighed in excess of 130 gm. at the time of introduction into the analytical respirometer. They were maintained in the experimental equipment for an adjustment period preceding each experiment. It is our impression that a minimum period of 5 days is desirable to establish satisfactory experimental conditions.

The animals were started on the experimental diet at least 2 weeks before observations were begun. The semisynthetic test diet, which was adequate to sustain pregnancy, growth, and lactation, was available to the experimental animals at all times. It had the following composition by weight:

Casein (vitamin-free)	200.0 gm.
Methionine	5.0
Choline	1.0
Sucrose	150.0
Starch	364.0
Crisco	240.0

[1] Received for publication August 1, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] This colony is a "closed colony" which has been maintained by parent-sibling, sibling-sibling breeding for more than 100 generations.

Cod-liver oil	10.0
Osborne-Mendel salts	40.0
	1,010.0 gm.

A vitamin supplement of the composition and amount described by Stekol and Weiss (*6*) was added to the diet.

The study was designed to allow comparison of the individual animal's metabolic performance obtained during a control period with that observed subsequent to tumor transplant and during ensuing tumor growth. A period of 8 to 12 days' duration was arbitrarily taken as an adequate control period. The induction period began with the aseptic transplant by trocar of a small piece of Walker carcinosarcoma 256 in the lateral flank of the experimental host and ended with the appearance of a minimally palpable lesion at the site of transplant. The experiment was terminated by killing the experimental host. No animal was kept until death occurred. Estimated weights of the growing neoplasms were obtained after the method of Schrek (*7*). Final tumor weights were obtained by actual weighing of the recovered tumor mass.

The experimental period for each animal consisted of continuous daily runs, from 21.5 to 23 hours' duration, throughout the designated number of consecutive days. During the time intervening between the end of one run and the beginning of the next, the food, water, drier, etc., were replenished and the experimental equipment was checked. For every run, the total oxygen, food and water consumption, and the total carbon dioxide, water vaporized, urine weight and total urinary nitrogen were measured. The weight of the daily wet and dry fecal mass was also measured as well as the total nitrogen content of the dry fecal mass.

Oxygen consumption and carbon-dioxide production volumes were extrapolated to a 24-hour daily value from the known duration of the run. All other measurements, for example, the food and water intake and the urinary and fecal nitrogen content, were assumed to represent daily values. It is recognized that this assumption will introduce daily errors because of variation in gut content; however, the prolonged experimental periods serve to reduce this error to a minimum.

Heats of combustion of food, dry urine, dry feces, dry carcass, and dry tumor were measured in the Parr adiabatic, oxygen bomb calorimeter. The nitrogen content of food, urine, feces, dry carcass, and dry tumor was obtained by semi-micro Kjeldahl technique.

Energy expenditure was calculated from the daily total volumes of oxygen used and carbon dioxide produced (corrected to standard conditions) and total urinary nitrogen using a form of Weir's equation (*8*):

$$\text{Total Kcal} = 3.815 \times \text{liters } O_2 + 1.232 \times \text{liters } CO_2 - 2.02 \times \text{gm. urinary N}$$

Carcass Analysis

All analyses for nitrogen, lipide, and caloric content were done on aliquots of dried carcass powder. Following decapitation and cleaning

of the gut, the carcass was ground and lyophilized, the loss of weight during lyophilization being taken as the water content of the carcass. The dried material was then ground 3 times in a Wiley mill. The excised tumors were similarly ground and lyophilized for analysis.

The lipide was extracted from carcass with 3 : 1 ethyl alcohol : diethyl ether and 1 : 1 chloroform : methanol after the method of Bloor and Haven (*9*). After evaporation of the extracts *in vacuo*, the material was redissolved in 4 : 1 petroleum ether : chloroform and washed with 50 percent ethyl alcohol. The petroleum ether solution was evaporated in a tared vessel *in vacuo* and the weight of the lipide determined. The 50 percent alcohol wash was concentrated *in vacuo* and added to the defatted material, which was then dried in a vacuum desiccator before analysis.

Results

The total body weights of the individual animals at the beginning of the experiment, at the time of transplant, and at the time of sacrifice are recorded in table 1. In table 2 the observed changes in total body weight, average daily weight gain, nitrogen retention, and caloric retention during the individual experimental periods have been tabulated. The data recorded during the respective control periods show that all animals were actively growing at the time of tumor transplant and were in positive nitrogen and caloric balance.

TABLE 1.—*Total body weight relations of rats receiving Walker 256 tumor transplant*

Rat No.	Total body weight			Tumor weight (*wet*)	Tumor weight
	Beginning	Transplant	Final		Final body weight
1390	268. 3	324. 1	404. 4	56. 0	. 14
1214	187. 2	228. 7	359. 0	74. 9	. 21
1964	183. 0	246. 5	264. 4	42. 0	. 16
1454	170. 0	215. 0	310. 5	Regressed	
1391	274. 4	302. 3	351. 0	Nontake	

Tumor transplant and tumor growth.—Three of the animals, rats #1390, #1214, and #1964, displayed a progressive growth of tumor following transplant of Walker 256 tumor tissue. A minimally palpable lesion appeared 6 to 9 days after transplant. Over a limited tumor period, the tumors showed 2 distinct phases of growth as estimated from external measurements—a relatively slow enlargement for 5 to 7 days followed by rapid growth. During the last 12 days of the prolonged tumor period of rat #1214, the tumor growth rate was substantially reduced, indicating a third growth phase.

Maximal growth rates of the individual tumors, taken from the steepest 3-day portion of the tumor growth curves, were approximately 9.1 gm. per day, 6.9 gm. per day, and 4.3 gm. per day for rats #1214, #1964, and #1390, respectively. The final weights of fresh tumor tissue recovered

TABLE 2.—*Changes in total body weight, average daily weight gain, nitrogen retention, and caloric retention*

Rat No.	Period	Days	△B. W. (*gm.*)	Avg. b. w. gain (*gm./day*)	N ret. (*gm.*)	Cal. ret. (*Kcal*)
1390	Control	12	55. 8	4. 6	1. 60	229. 74
	Induction	9	27. 5	3. 0	. 78	136. 53
	Tumor	19	52. 8	2. 8	1. 20	206. 39
1214	Control	8	41. 5	5. 2	1. 08	99. 91
	Induction	6	23. 3	3. 9	. 81	39. 25
	Tumor	36	107. 0	2. 9	3. 29	102. 34
1964	Control	8	46. 8	5. 8	1. 35	140. 95
	Induction	6	22. 2	3. 7	. 79	58. 59
	Tumor A	12	43. 4	3. 6	1. 23	75. 82
	Tumor B	7	−46. 8	−6. 6	−1. 23	−248. 68
1454	Control	9	45. 0	5. 0	1. 47	114. 63
	Induction	8	40. 0	5. 0	1. 36	84. 08
	Tumor	9	34. 5	3. 8	1. 13	49. 79
	Regression	12	18. 7	1. 6	. 91	32. 59
1391	Control	9	31. 8	3. 5	. 85	96. 21
	Posttransplant	18	48. 7	2. 7	1. 09	290. 28

from the individual animals are recorded in table 1 as well as the relation of the final tumor weight to the total body weight at the time of killing the animals.

Transplant of tumor tissue into rat #1454 resulted in the appearance of tumor that attained a calculated weight of 9.7 gm. before undergoing a complete and spontaneous regression. No evidence of tumor growth after transplant was observed in rat #1391 during the entire 18-day post-transplant period.

Tumor analysis.—The dry weight and water content of the recovered tumor tissue from the tumor-bearing animals were determined. The dry tumor was analyzed for nitrogen content and caloric content. The results, expressed as total values, are given in table 3.

TABLE 3.—*Tumor analysis**

Rat No.	Dry weight (*gm.*)	Water weight (*gm.*)	N content (*gm.*)	Calories (*Kcal*)
1390	8. 4	47. 6	. 99	44. 77
1214	12. 4	62. 5	1. 46	66. 09
1964	6. 0	36. 0	. 71	31. 98

**Factors:* 5.33 ± 0.04 Kcal per gm. dry weight; 11.8 ± 0.1 percent N per gm. dry weight.

The analytical studies revealed only minimal variation in composition among the tumors. Water content varied from 83.4 to 85.7 percent. The nitrogen content per gm. of dry tumor tissue was 11.8 ± 0.1 percent and the caloric content was 5.33 ± 0.04 Kcal per gm. of dry tissue.

Carcass analysis.—The results of the analyses to determine gross composition of the animal's carcass (total animal free of tumor) at the termina-

TABLE 4.—*Carcass analysis*

Rat No.	Carcass				Defatted carcass		R*
	Dry weight (*gm.*)	Water (*gm.*)	Percent N (*dry wt.*)	Percent F (*dry wt.*)	Weight (*gm.*)	N (*percent*)	
1390	157. 5	184. 5	6. 7	49. 0	80. 3	13. 0	2. 3
1214	88. 0	192. 1	8. 7	33. 0	58. 9	13. 0	3. 3
1964	61. 0	160. 0	9. 7	25. 7	45. 3	13. 1	3. 5
1454	107. 0	197. 5	8. 3	37. 0	67. 4	13. 1	2. 9
1391	130. 0	216. 0	6. 9	48. 0	68. 5	13. 2	3. 1

*R is the ratio of grams of water to grams of defatted, dry carcass.

tion of the experiment are recorded in table 4. There was wide variation among the animal carcasses. Water content ranged from 53.9 to 72.4 percent. The percentage of nitrogen in dry carcass varied from 6.7 to 9.7 and the percentage of fat varied from 25.7 to 49.0. However, the dry, fat-free carcass showed minimal variation in nitrogen content (13.1 $\pm$ 0.1%) and caloric content (4.55 $\pm$ 0.08 Kcal/gm.), a result consistent with the concept of a chemically mature rat (*10–13*). The extracted body fat averaged 9.26 $\pm$ 0.03 Kcal per gm.

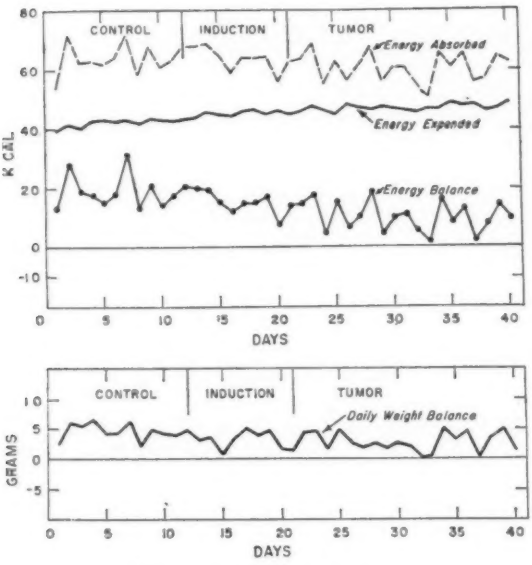
The ratio (R) of the grams of water in total carcass to grams of dry, fat-free carcass has been calculated as an index of relative hydration of the animals at the termination of the experiment (table 4). According to the values in the literature (*10, 11, 14*) and a limited number of our own observations on normal animals in this weight range, the average water content of fat-free carcass is 73.1 to 75.5 percent, corresponding to ratios of 2.7 to 3.1. The variation in this ratio for the experimental animals indicates an increase in relative carcass hydration for rats #1214 and #1964, and a significant carcass dehydration in rat #1390. There would appear to be minimal alteration of carcass hydration in rats #1454 and #1391.

Energy Balance

Food Intake.—The daily values for food intake were variable. This can be seen in the plots of energy absorbed (ingested energy minus fecal energy) by the individual animals over the total experimental periods (text-figs. 1–5). Much of this variation is undoubtedly due to the day-to-day change in gut content with consequent error appearing in the apparent ingested energy and absorbed energy values.

In table 5 the average daily values for grams of food eaten in the respective experimental periods have been recorded. The data show that the tumor-bearing animals decreased their food intake during the induction and tumor periods below the levels observed during the respective control periods (text-figs. 1–3). Rats #1390 and #1214 experienced only moderate reduction and rat #1964 ultimately suffered complete appetite loss.

The regressor animal, rat #1454, increased food intake in the induction period and tumor period compared to the control-period level but had a

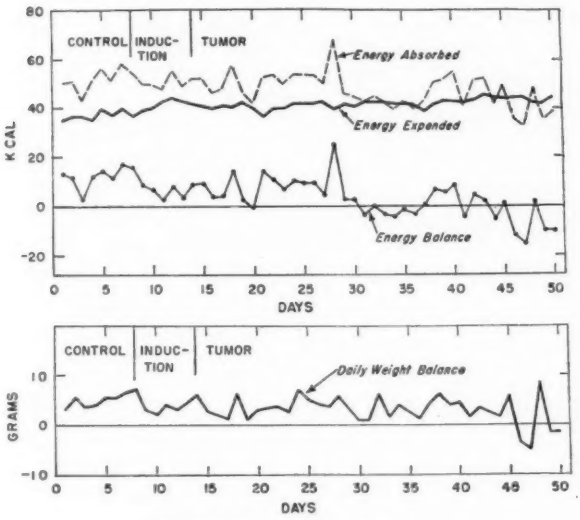


TEXT-FIGURE 1.—Rat #1390.

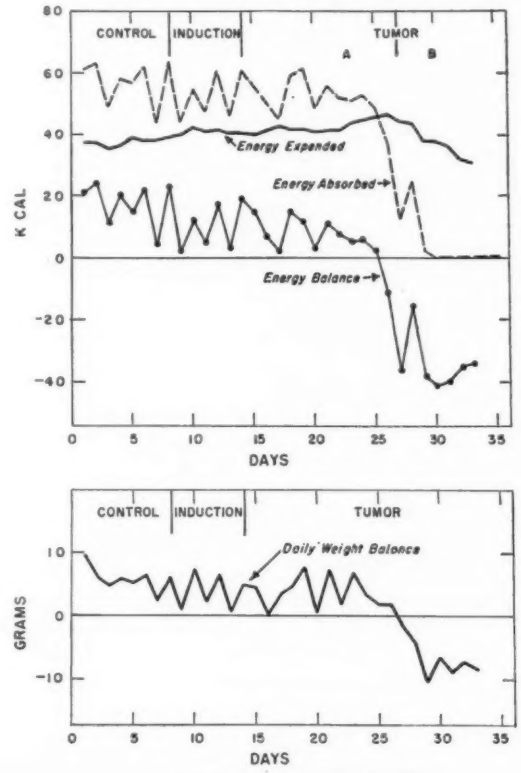
TABLE 5.—*Observed changes in food intake and energy expenditure*

Rat No.	Period	Food intake (*gm./day*)		Total energy loss (*Kcal/day*)		Energy expenditure (*Kcal/day*)	
		Average	Percent of control	Average	Percent of control	Average	Percent of control
1390	Control	13. 25	100	51. 27	100	42. 43	100
	Induction	13. 27	100	55. 20	108	45. 57	107
	Tumor	12. 62	95	56. 67	111	47. 24	111
1214	Control	11. 08	100	45. 66	100	37. 56	100
	Induction	10. 96	99	50. 96	111	41. 93	111
	Tumor	9. 94	90	49. 30	108	42. 37	113
1964	Control	11. 90	100	44. 91	100	38. 57	100
	Induction	11. 05	93	48. 23	107	40. 99	106
	Tumor A	10. 82	91	50. 57	113	42. 84	111
	Tumor B	1. 05	09	41. 25	92	37. 89	98
1454	Control	10. 29	100	41. 28	100	35. 02	100
	Induction	11. 17	109	47. 00	114	39. 93	114
	Tumor	11. 01	107	49. 83	121	42. 22	121
	Regression	9. 67	94	49. 09	119	42. 38	121
1391	Control	10. 87	100	46. 41	100	38. 31	100
	Post-transplant	12. 00	110	46. 83	101	38. 91	102

reduced food intake in the regression period (table 5 and text-fig. 4). The nontake animal, rat #1391, substantially increased food intake during the post-transplant period (table 5 and text-fig. 5).



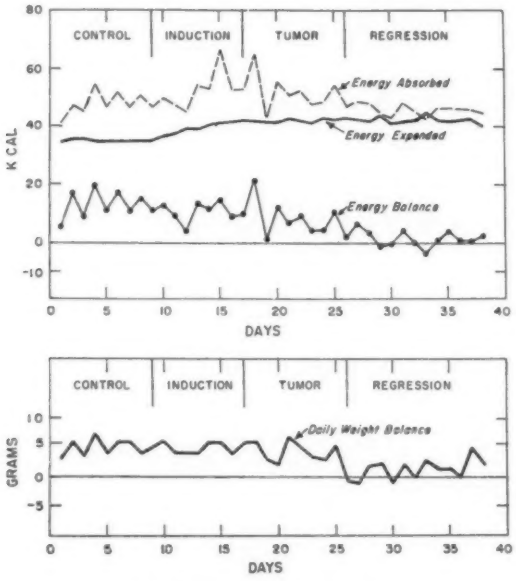
TEXT-FIGURE 2.—Rat #1214.



TEXT-FIGURE 3.—Rat #1964.

Total energy loss.—An abrupt rise in total energy loss above control-period levels was seen following tumor transplant in all animals that subsequently showed tumor growth (table 5). The rise during the induction period for all animals varied between 7 and 14 percent of the control-period average. Higher levels of total energy loss, up to 108 to 121 percent of the control-period average, were observed during the tumor period as long as the appetite was sustained. Only a minimal rise in total energy loss was observed in rat #1391.

Energy expenditure.—It is apparent from the data in table 5 that the increase in an animal's total energy loss was related almost entirely to increased energy expenditure. Inspection of the daily energy-expenditure curves of the individual animals (text-figs. 1–4) reveals that the in-



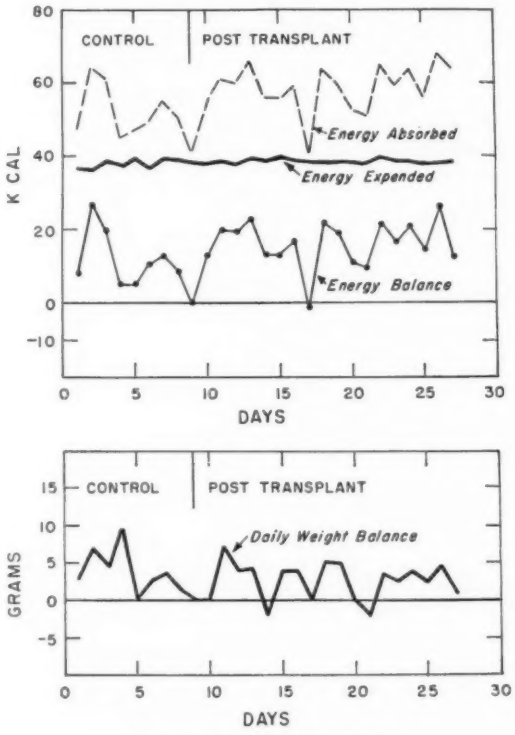
TEXT-FIGURE 4.—Rat #1454.

crease followed closely upon tumor transplant and was apparent before the appearance of any palpable tumor mass. The daily energy expenditure attained maximum values during the tumor period when the level of food intake was sustained. It is of interest to note that the level of energy expenditure of rat #1454 remained elevated during the regression period (table 5). The nontake animal, rat #1391, showed only a minimal increase in daily energy expenditure in the post-transplant period.

Energy balance.—The net caloric balances for the individual animals during the experimental periods are recorded in table 2. The daily energy balance of the individual animals is shown graphically in text-figures 1–5. It may be seen that all animals showed an adequate, positive energy balance in their control periods.

The tumor-bearing animals, rats #1390, #1214, and #1964, remained in positive caloric balance during their induction periods; however, the level of daily energy retention was decreased from control-period values (table 2 and text-figs. 1–3). This decrease reflects the increased energy expenditure and total energy loss seen in this period in the absence of any increase in food intake.

During the tumor periods, the daily energy retention of all tumor-bearing animals continued to fall (text-figs. 1–3), reflecting the further increase in energy expenditure and the diminishing level of food intake



TEXT-FIGURE 5.—Rat #1391.

(table 5). Rat #1390 showed a net positive caloric balance for the tumor period (table 2); no negative daily energy-balance values were observed (text-fig. 1). Rat #1214 had a net positive caloric balance for the tumor period (table 2); however, negative daily energy-balance values were observed on several experimental days (text-fig. 2).

The tumor period of rat #1964 has been divided arbitrarily into periods A and B to show the effects of the abruptly occurring anorexia. The animal remained in net positive caloric balance in tumor period A but the level of daily energy balance progressively decreased from control-period

levels. During the period of complete appetite loss the animal was in marked negative daily energy balance (text-fig. 3) and sustained a severe net caloric deficit (table 2), even though there was a significant reduction in energy expenditure (text-fig. 3) and in total energy loss (table 5) during this period.

Rat #1454 remained in positive caloric balance throughout the entire experimental period (table 2). During the induction period, the daily level of energy retention showed only minimal change from control period levels because an increased food intake apparently offset the increased energy expenditure which occurred (table 5). Maximal levels of energy expenditure (121% of control-period level) were observed in the tumor and regression periods (table 5). The food intake diminished from the high values of the induction period, resulting in a progressive fall in the level of daily energy retention (text-fig. 4).

Rat #1391 showed an increasing daily energy retention in the post-transplant period (text-fig. 5) primarily related to a substantial increase in food intake with only minimal increase in total energy loss (table 5).

Total Body Weight

All animals showed a rapid rate of growth and weight gain during their control periods (tables 1 and 2).

The average daily weight gain of the tumor-bearing animals, rats #1390, #1214, and #1964, showed a marked reduction from control period values during their induction and tumor periods (table 2). The progressive decrease in the total daily weight gain during the induction and tumor periods is readily apparent from inspection of the plots of daily weight balance for the individual animals (text-figs. 1–3). Presumably this result reflects the increased energy expenditure and decreased food intake and thus decreased energy retention experienced by these animals during their induction and tumor periods.

Rat #1454 maintained, during the induction period, levels of average daily weight gain and energy retention comparable to those observed in the control period (table 2). There was reduction in the average daily weight gain during the tumor and regression periods that appears to be related to the decreasing energy retention of these periods (text-fig. 4).

Rat #1391 presents the interesting finding of a decreased average weight gain in the post-transplant period (table 2). During this period a significant increase in food intake occurred with only minimal increase in total energy loss (table 5). This result of increased energy retention per gram of body weight added, suggests that a significant shift occurred in the proportions of the kinds of body weight added during this period.

Carcass Weight

The calculated changes in carcass weight and composition of the individual animals during the experimental periods are recorded in table 6. These calculations were based on the observed weight change, the energy

balance data, and the results obtained from carcass and tumor analyses. In addition, the following values were used as constants:

1) 1 gm. of nonfat body weight (NF)=13.1 percent N
=4.55 Kcal

and

2) 1 gm. of fat body weight (F)=9.26 Kcal

In the calculations it has been assumed that the changes in the animal's weight, nitrogen balance, and caloric balance during the control and induction periods reflect change only in the animal carcass. To determine the changes in the animal's carcass during the tumor period, the total weight, total nitrogen content, and total caloric content of the individual tumor has been subtracted from the measured change in these parameters for the whole animal during the tumor period.

The calculated values of grams of fat and nonfat body weight represent dry weight. The change in water content of the carcass was considered to be the difference between the observed change in total body weight of the animal (corrected for tumor weight in the tumor period) and the sum of the calculated dry fat and nonfat body weights.

The calculations reveal that approximately 50 percent of the dry carcass weight gained by rat #1964 during the control period was fat; this proportion decreased to 35 percent in the induction period. A carcass weight loss of 45.3 gm. occurred in the total tumor period (A and B). During the early tumor period, the animal was in positive weight, nitrogen and caloric balance. The loss of appetite and the continued rapid growth of the tumor during the final days of the experiment resulted in an over-all weight loss, nitrogen loss, and calorie loss from the carcass. Approximately equal amounts of fat and water comprised the major portion of this weight loss, with a few grams of nonfat body weight accounting for the remainder.

Rat #1214 showed a net gain in carcass weight in all experimental periods. The calculated gain in carcass fat was approximately 45 percent of the dry weight in the control period and 16 percent in the induction period. In the tumor period the carcass weight added was only nonfat weight and water; there was a small net loss of carcass fat.

During the control period, rat #1390 added 31.0 gm. of dry carcass weight of which 60 percent was fat. Similar proportions of dry weight gained (62 percent fat) were maintained in the induction period. During the tumor period the animal exhibited a minimal loss of carcass weight (3.2 gm.) but never showed any negative values for total weight balance or daily energy balance. Nitrogen and calories were retained in excess of the amounts recovered from the tumor (tables 2 and 3). The dry-weight gain of the carcass in this period was 18.3 gm. of which 16.7 gm. or 91 percent was fat (table 6). The increase in dry weight, together with the net carcass weight loss, indicates the loss of 21.5 gm. of water from the carcass. This calculated shift in carcass composition is in agreement with the values obtained from carcass analysis (table 4), *i.e.*, high fat content and relative dehydration.

TABLE 6.—*Calculated changes in carcass composition based on the measured values of total body-weight difference, tumor weight, nitrogen balance, and caloric balance*

Rat No.	Period	Weight diff. (*gm.*)	N Ret. (*gm.*)	E Ret. (*Kcal*)	NF Wt. (*gm.*)	NF Cal. (*Kcal*)	F Cal. (*Kcal*)	F Wt. (*gm.*)	W Wt. (*gm.*)
1390	Control	55. 8	1. 60	229. 74	12. 2	55. 51	174. 23	18. 8	24. 8
	Induction	27. 5	. 78	136. 53	6. 0	27. 30	109. 23	11. 8	9. 7
	Tumor	−3. 2	. 21	161. 62	1. 6	7. 28	154. 34	16. 7	−21. 5
1214	Control	41. 5	1. 08	99. 91	8. 2	37. 31	62. 60	6. 8	26. 5
	Induction	23. 3	. 81	39. 25	6. 2	28. 21	11. 04	1. 2	15. 9
	Tumor	32. 1	1. 83	36. 25	14. 0	63. 70	−27. 45	−3. 0	21. 1
1964	Control	46. 8	1. 35	140. 95	10. 3	46. 87	94. 08	10. 2	26. 3
	Induction	22. 2	. 79	58. 59	6. 0	27. 30	31. 29	3. 4	12. 8
	Tumor A and B	−45. 4	−. 71	−204. 72	−5. 4	−24. 57	−180. 15	−19. 4	−20. 6
1454	Control	45. 0	1. 47	114. 63	11. 2	50. 96	63. 67	6. 9	26. 9
	Induction	40. 0	1. 36	84. 08	10. 4	47. 32	36. 76	4. 0	25. 6
	Tumor-reg	53. 2	2. 04	82. 38	15. 6	70. 98	11. 40	1. 2	36. 4
1391	Control	31. 8	. 85	96. 21	6. 5	29. 58	66. 63	7. 2	18. 1
	Posttransplant	48. 7	1. 09	290. 28	8. 3	37. 77	252. 51	27. 2	13. 2

It is of interest to note that the tumor water content was 85 percent (table 3) and thus did not show any evidence of the dehydration seen in the carcass of this animal.

The regressor animal, rat #1454, showed a positive weight, nitrogen and caloric balance for the entire experimental period. It can be seen from table 6 that the proportion of fat added to the carcass progressively decreased with a corresponding increase in nonfat weight and water content.

During the control period the dry weight gained by rat #1391, the nontake animal, was 53 percent fat. This proportion increased to 77 percent following transplant.

Discussion

The animals presented in this experimental series were selected to show the variations in total metabolism, and particularly energy metabolism, which have been observed following the transplant of Walker 256 tumor in the rat.

The common observations that have characterized all our studies on the progressive growth of this tumor in the Osborne-Mendel strain of rat were: 1) an increasing total energy expenditure, and 2) a diminishing food intake during the tumor period compared to control period levels.

The increase in total energy expenditure followed almost immediately after tumor-tissue transplant and was certainly manifest before any palpable tumor mass was present. The rather sudden initial rise seen in the induction period was usually followed by a continued, slower increase in the total energy expenditure during the tumor period.

Many physiological factors could contribute to the increased total energy expenditure of the induction period. The Walker 256 tumor is probably an extremely anaplastic or undifferentiated adenocarcinoma of the breast that has been modified by numerous passages through various hosts. An immunochemical response could be involved as could many other factors, such as the age and weight of the animal at transplant or the amount of tumor tissue implanted.

It is of interest to note that rat #1391 showed no increase in total energy expenditure immediately after transplant and only a minimal increase over the entire post-transplant period. This would suggest that the presence of a small piece of tumor tissue and the traumatic effect of the trocar are of little importance and that the energy response is related primarily to the "viability" of the transplant. It must be pointed out, however, that rat #1391 is the only nontake animal that we have had the opportunity to study.

Total energy expenditure is made up of 3 major components, *i.e.*, the resting basal energy cost, the energy cost of movement and activity, and the energy cost of food utilization. The present studies suggest that in the presence of growing tumor the resting basal-energy cost is increased, but the data from this series are insufficient to determine the contribution of each of the components to the rise in total energy expenditure. For example, rat #1964 became very inactive and had essentially zero food intake during the last 7 days of the tumor period. During this period,

there was a rapid loss of carcass weight and a sharp fall in total oxygen consumption and total energy expenditure (text-fig. 3). However, rapid tumor growth continued. Study of the resting levels of oxygen consumption in this period showed values approximately equal to the resting values for oxygen consumption in the pre-anorexia tumor period, suggesting that the decrease in energy expenditure seen was the result of decreased energy cost for activity and food utilization.

It has been reported by Mider *et al.* (*15*) that rats experiencing progressive growth of Walker 256 tumor contain less total lipide (carcass plus tumor) than do their pair-fed controls. Haven *et al.* (*16*) have reported a similar result and, additionally, have shown the decrease in total lipide content to be a decrease in the fatty-acid fraction. As pointed out by Mider (*17*), since the pair-fed control and the tumor-bearing animals eat identical amounts of food, the difference in total lipide content between such animals could not result from decreased food intake. The difference would seem to reflect an increased total energy expenditure of the tumor-bearing animals.

The observations reported here may constitute a satisfactory mechanism to explain the difference in total lipide content between a tumor-bearing animal and its pair-fed control. Such a difference could develop whether the animals were in positive or negative caloric balance. In the former case, the increased energy expenditure of the tumor bearer represents the loss of potential lipide for storage; in the latter, an even more rapid depletion of body lipide.

An increased metabolic rate and decreased energy retention cannot be considered specific for the tumor process. Neither do the alterations in the amount and composition of body lipides of the tumor-bearing host appear to be specific for tumor growth.

Our data indicate that among rats receiving a constant diet and growing a tumor of almost constant composition, wide variations can occur in gross carcass composition. Future work in energy metabolism must be directed toward correlation of the changes that occur in carcass composition during tumor growth with alterations that presumably occur in the energy-yielding reactions of the host, particularly as these reactions are effected by the host's caloric requirements and the availability of essential nutrients.

Summary

The implantation and subsequent growth of Walker carcinosarcoma 256 tumor in the Osborne-Mendel strain of rat is associated with an increased total energy expenditure, a progressive reduction in food intake, and, therefore, a progressive decrease in energy retention. The alterations in energy metabolism are reflected as significant changes in the gross composition of the tumor-bearing animals.

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Long-Term Study of X-Irradiated Rabbits with Bone-Marrow Homotransplants [1,2]

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Previous studies by numerous workers (*1–3*) have established that effects of total-body X irradiation on mice, rats, hamsters, and guinea pigs may be modified by bone-marrow injections. Furthermore, it has been shown that this modification is dependent upon the survival of injected marrow cells and their repopulation of the host's depleted hematopoietic tissues (*4–9*).

Recently, similar findings were reported in the rabbit (*10*). In this study the morphological difference between male and female polymorphonuclear leukocytes was used as a biological cell marker to indicate survival of transplanted female marrow cells in irradiated male animals. The relative sensitivity of this species to X irradiation produced a high mortality during the initial shocklike state and, later, from gastric ulceration and perforation. This high mortality made observations on the long-term survival of the injected marrow difficult.

The present paper records a lower incidence of these complications which was accomplished by giving the total-body X irradiation in divided dosage and by administering an antibiotic for the first 3 weeks after irradiation. With the resulting reduction in early deaths, it has been possible to utilize the cell-marker technique for studying, over a prolonged period, a number of rabbits that survived otherwise lethal X irradiation followed by homologous marrow transplantation.

Materials and Methods

Young adult male New Zealand white rabbits, which were not inbred in the genetic sense, weighing 2.5 to 3 kg., were exposed to total-body X irradiation from a Phillip's machine under the following conditions: 250 kvp, 15 ma., 50 cm. target distance, 0.4 mm. Sn, 0.25 mm. Cu and 1.0 mm. Al filters, HVL-2.5 mm. Cu, 50 r per minute dose rate. The dosage

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TABLE 1.—*Effect of tetracycline and homologous bone-marrow therapy on survival of rabbits receiving 1,100 r total-body X irradiation, fractionated into two doses*

Group	Experimental conditions	No. rabbits	Percent survivors after weeks: 1	4	8	16	32
1	X ray + marrow + antibiotic	40	95	62	42	40	40
2	X ray + antibiotic	20	95	35	30	25	25
3	X ray alone	20	90	15	10	10	10

was divided: An initial dose of 600 r was followed 24 hours later by an additional 500 r.[6]

The animals were then divided into 3 groups (table 1).

Group 1 consisted of 40 rabbits which were given, approximately 1 hour after the last irradiation, a single intravenous injection of a saline suspension of fresh bone marrow from a female rabbit. The volume was adjusted so that each rabbit received 10 ml. of a suspension containing 1,200 ($\times 10^6$) nucleated cells. Starting on the day of the last irradiation they were also given tetracycline hydrochloride[7] in their drinking water. The concentration was adjusted so that each animal drank 70 to 75 mg. of tetracycline per day. Any animal failing to drink, or drinking very little, received supplemental intramuscular tetracycline to ensure the same total dose of antibiotic. Tetracycline therapy was continued for 3 weeks.

The 20 irradiated rabbits in group 2 were given tetracycline hydrochloride following irradiation, but no bone marrow.

The third group of 20 rabbits received neither antibiotic nor bone marrow after irradiation.

Total white-cell counts and differentials, hemoglobins, and microhematocrits were taken on the irradiated animals every 2 days for the first 30 days and once a week thereafter. At the same intervals blood films were taken from the ear, the smears were stained by Wright's method, and the heterophils were examined for nuclear "drumsticks," as described previously (*10*). Before accepting the fact that the leukocytes were at least partially derived from the transplanted female bone marrow, it was again arbitrarily decided that a minimum of 6 cells showing typical discrete chromatin nodules should be found. The number of such cells seen in examining 300 heterophils was recorded as a percentage of the heterophil count.

The rabbits were weighed daily for the first month, then at weekly intervals. At death all animals were autopsied, but the brain was not examined. Marrow from the midpoint of the shaft of the right femur and tissue from the thymus, spleen, mesenteric lymph node, and appendix were taken and fixed in Helly's fluid. Other tissues were fixed in formol-

[6] We are indebted to Dr. G. D'Angio and the Children's Hospital X-ray Department for granting us radiation facilities.

[7] Achromycin®, soluble in water, was kindly furnished by Lederle Laboratories Division, American Cyanamid Company.

saline. All were routinely stained with hematoxylin and eosin. Marrow smears were prepared by the method of Berenbaum (*11*) and treated with Wright's stain.

Results

Group I (X Ray + Marrow + Antibiotic)

The results for this group of 40 male rabbits are summarized in table 2.

Died immediately after irradiation.—Two animals (5%) died in a state of shock within 12 hours following the initial dose of 600 r whole-body X irradiation. At autopsy, pulmonary congestion and hemorrhage and petechial hemorrhages into the thymus and other lymphoid tissue were seen.

Unsuccessful marrow transplant.—Twelve animals (30%) failed to show female leukocytes bearing nuclear "drumsticks" in any of the peripheral-blood smears obtained after irradiation and it was concluded that the

TABLE 2.—*Analysis of effects on 40 male rabbits of 1,100 r total-body X irradiation fractionated into two doses followed by homologous bone marrow and tetracycline*

Effect	No. rabbits	Percent
Died in shock immediately after irradiation	2	5
Failed to show successful marrow transplant	12	30
Died from gastric perforation at 12 and 13 days	2	16.8
Died from infection at 13–25 days	5	41.6
Regenerated own bone marrow.		
Survived	4	33.3
Died at 91st day	1	8.3
Showed successful marrow transplant	26	65
Died from gastric perforation at 10–15 days	4	15.5
Died at 23 and 25 days as result of early rejection of marrow transplant by host	2	7.7
Died from infection at 33–40 days with marrow transplant intact	7	27.0
Marrow transplant rejected by host at 38–46 days:		
Survived	2	7.7
Died at 44th day	1	3.9
Remained alive and well with marrow transplant intact	10	38.2

attempt at bone-marrow homotransplantation had been unsuccessful. Two of these rabbits subsequently died from perforation of a gastric ulcer at 12 and 13 days, respectively, after the last dose of irradiation. Examination of the bone marrow of one rabbit showed early regeneration from its own hematopoietic tissues; no female cells were found. The other still showed complete marrow aplasia.

Five failed to regenerate their own bone marrow. The total white-cell counts remained in the 500 to 1,000 per mm.^{3} range, and these animals died at 13 to 25 days. At autopsy, all showed evidence of bacteremia, nonpurulent infection, and bone-marrow aplasia.

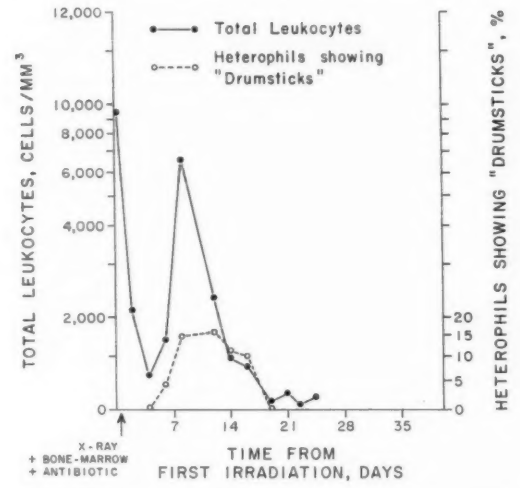
Five rabbits, however, did regenerate their own bone marrow; the total white-cell counts began to rise in 14 to 16 days, and reached normal levels at 21 to 28 days. No female cells were ever seen. Four rabbits survived the 32 weeks of the experiment; one was killed when moribund at 13 weeks. The latter animal was killed after developing diarrhea, with a leukocyte count of 13,000 cells per mm.^{3} for 3 weeks, and progressive loss of weight from 2,970 to 1,700 gm. in 5 weeks. At autopsy, the bone marrow was hyperplastic, no female cells were found, and the lymphoid tissues had returned to normal with the exception of the thymus, which was incompletely regenerated, and there was hemosiderin in the spleen. A small gastric carcinoma, bronchopneumonia, and intravascular clumps of bacteria were also present. The four survivors all showed a total white-cell count of about 10,000 per mm.^{3} with numerous periods of leukocytosis up to 20,000 cells per mm.^{3} The initial body weight was never completely regained, fur loss over head and back was permanent, and cataracts occurred in two rabbits.

Successful marrow transplant.—Twenty-six rabbits (65%) showed a rapid rise in the total white-cell count at 3 to 5 days and reaching 4,000 to 8,000 cells per mm.^{3} at 9 days. At the same time significant numbers of heterophils with characteristic nuclear "drumsticks" appeared in the peripheral blood, which indicated persistence and function of the homotransplanted marrow.

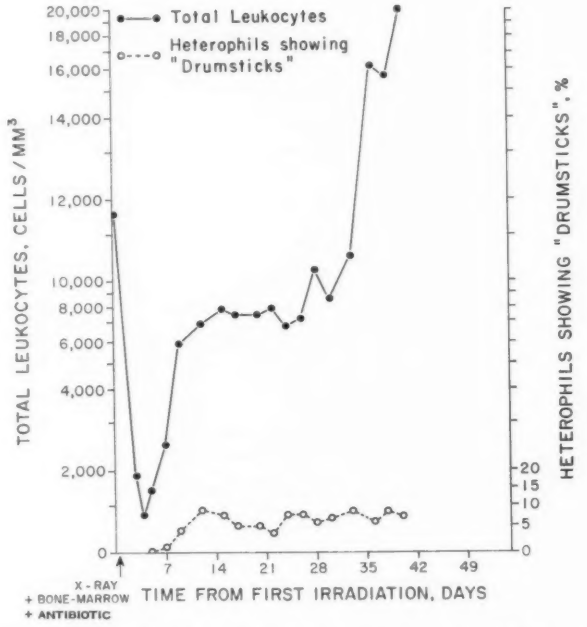
Four of these animals died from perforation of a gastric ulcer or gastric rupture at 10 to 15 days. At autopsy, the bone marrow was hyperplastic and smears showed female cells. Restoration of the lymphoid tissues was occurring.

Two died at 23 and 25 days as a result of early rejection of the marrow transplant by the host (text-fig. 1). In both these rabbits there was an initial rapid rise in the total white-cell count to 6,500 and 7,000 cells per mm.^{3}, respectively, by the 8th day, and 10 to 15 percent of the heterophils showed characteristic female sex chromatin. By the 12th day, the total white-cell count had fallen to about 2,300 cells per mm.^{3}, and this sharp decline continued to about 200 cells per mm.^{3} on the 19th day, and then remained below 500 cells per mm.^{3} until death occurred. Simultaneously, the percentage of female heterophils declined until the 18th day when all had disappeared. The hematocrit packed-cell volume fell from an initial value of about 43 percent to 41 percent at the 12th day and then to 22 percent at the 20th day. *Post mortem*, there was marked destruction of the bone marrow with only a few tiny islands of remaining hematopoietic cells (fig. 2, *cf.* fig. 1). No female cells were found in smears. There was marked regeneration of the lymphoid nodules in the spleen and appendix (fig. 6); the thymus, however, was still largely depleted of lymphocytes. A nonperforated gastric ulcer was seen at the pylorus in one of the two cases, and the lungs of both showed extensive bronchopneumonia.

Seven rabbits died at 33 to 40 days as a result of infection (text-fig. 2). These rabbits had a successful marrow homotransplant as shown by rapid



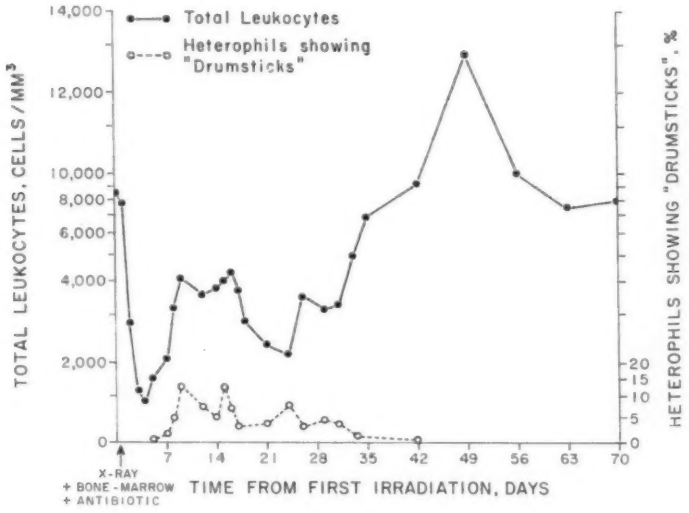
TEXT-FIGURE 1.—Total leukocyte count and percentage of heterophils showing female sex chromatin in X-irradiated male rabbit R70 treated with homologous bone marrow and tetracycline.



TEXT-FIGURE 2.—Total leukocyte count and percentage of heterophils showing female sex chromatin in X-irradiated male rabbit R54 treated with homologous bone marrow and tetracycline.

restoration of the total white-cell count and appearance of female heterophils. Nevertheless, at about the 28th day a rapid loss of body weight occurred, which was associated with diarrhea and a leukocytosis of 18,000 to 20,000 cells per mm.^{3} The percentage of heterophils showing female sex chromatin, however, was unaltered and the hematocrit packed-cell volume and hemoglobin remained close to their pre-irradiation values. At autopsy, the bone marrow appeared normal or hyperplastic (fig. 3) and female cells could be demonstrated in smears (fig. 4). The splenic lymphoid nodules and other lymphoid tissue showed little regeneration, being markedly atrophic with destruction of the germinal centers resulting in prominence of the reticular cells (fig. 7). In the spleen, hemosiderin containing macrophages were common in the red pulp; groups of plasma cells at the sites of the lymphoid nodules occurred in some, and extensive hemorrhages with areas of necrosis were in the red pulp of one animal. In two rabbits, the thymus showed some lymphoid regeneration; in the others it remained completely atrophic. Extensive bronchopneumonia was common to all and histological evidence of gut infection was seen in four.

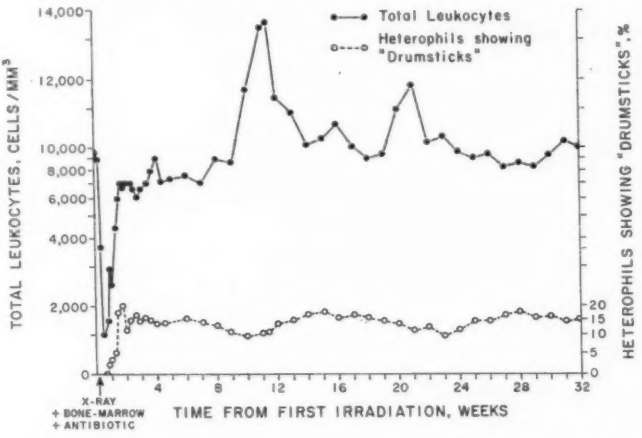
In three rabbits there was late rejection of the marrow transplant between 38 and 46 days (text-fig. 3). After rapid restoration of the total white-cell count to 4,000 to 5,000 cells per mm.^{3} by the 9th day, a drop in the count started at about the 16th to 18th day reaching 2,000 to 2,500 cells per mm.^{3} at the 24th to 28th day. Female heterophils continued to be found during this period, but the percentage declined steadily. From about the 28th day the total white-cell count in two rabbits rose steadily



TEXT-FIGURE 3.—Total leukocyte count and percentage of heterophils showing female sex chromatin in X-irradiated male rabbit R49 treated with homologous bone marrow and tetracycline.

reaching 9,000 per mm.^{3} at about the 42nd day, but female heterophils diminished steadily and finally disappeared at about this time. One animal did not show this restoration of the leukocytes and died on the 44th day with a white-cell count of 500 per mm.^{3} At autopsy, the marrow was aplastic, and bronchopneumonia was the immediate cause of death. The lymphoid tissue of the spleen, appendix, and mesenteric lymph node appeared normal. The thymus was incompletely restored. The two survivors retained a white-cell count about normal levels, and at times showed a leukocytosis of 16,000 to 20,000 cells per mm.^{3} Hematocrit and hemoglobin values, after falling at the time the marrow transplant was being rejected, rose later to pre-irradiation levels in the two survivors.

Ten animals showed a successful marrow homotransplant (text-fig. 4), and after the initial weight loss regained their original weight. Apart



TEXT-FIGURE 4.—Total leukocyte count and percentage of heterophils showing female sex chromatin in X-irradiated male rabbit R51 treated with homologous bone marrow and tetracycline.

from loss of fur in all and cataract in three, they remained alive and well for the 32 weeks of the experiment. During this period, the total leukocyte count remained at, or above, normal levels and continued to show a constant percentage of female heterophils. Periods of leukocytosis, often accompanied by temporary weight loss, occurred in these as in the other groups of survivors. The hematocrit and hemoglobin values never changed appreciably from the pre-irradiation levels.

The cumulative mortality in animals of group 1 is shown in table 3.

Group 2 (X Ray + Antibiotic)

Of the 20 animals in this group:

a) One (5%) died within 12 hours following the initial dose of total-body X irradiation.

TABLE 3.—*Cumulative mortality in male rabbits given 1,100 r whole-body X irradiation fractionated into two doses followed by homologous bone marrow and tetracycline (group 1)*

Experimental group	No. rabbits	Percent mortality after weeks: 1	2	3	4	5	6	7	8	16	32
Animals with successful marrow transplant	26	—	12	15	23	31	50	54	54	54	54
Animals in which marrow transplant failed	12	—	25	50	58	58	58	58	58	67	67
All animals in group 1	40	5	20	30	38	43	55	58	58	60	60

b) Three (15%) died from perforation of a gastric ulcer at 10 to 15 days after the final dose of irradiation.

c) Seven (35%) regenerated their own bone marrow, as evidenced by late restoration of the leukocyte count. Five rabbits survived the experimental period, but two died at 40 and 80 days, respectively, from infection. Microscopically, normal restoration of the hematopoietic and lymphoid tissues seemed complete.

d) Nine (45%) died of infection, mainly bronchopneumonia, at 14 to 28 days without regenerating their own bone marrow.

Group 3 (X Ray Alone)

Of the 20 animals in this group:

a) Two (10%) died immediately after the irradiation.

b) Five (25%) died following perforation of a gastric ulcer or gastric rupture at 10 to 17 days.

c) Three (15%) regenerated their own bone marrow. One rabbit subsequently died from infection at 26 days but two survived the entire period of the experiment.

d) Ten (50%) died from infection at 8 to 20 days without showing evidence of regeneration of their own bone marrow. Seven of these deaths occurred before the 14th day.

Table 1 shows the percentage of animals surviving in each of these experimental groups after various intervals of time.

Discussion

Until recently, the mechanism by which bone-marrow treatment induces rapid hematopoietic recovery in lethally irradiated rodents was a matter of contention. Two hypotheses were favored: "humoral" stimulation of surviving cells leading to regeneration of the host marrow and "cellular repopulation" of the depleted host marrow by the injected donor cells. However, several reports appeared almost simultaneously with direct evidence of survival and proliferation of transplanted marrow cells in irradiated recipients (*4–9*).

With the acceptance that cellular repopulation is the main mechanism by which marrow brings about its therapeutic effect, attention has turned

to the nature of the late mortality that occurs in irradiated animals treated with homologous or heterologous bone marrow. In the present experiments with homologous bone marrow, an 80 percent survival at 2 weeks was reduced to 42 percent by 7 weeks.

Possible explanations of this late mortality are that it represents a delayed immunological response of the host to the foreign-strain marrow (*12*) or that it is the result of a transplanted homologous antibody-producing system reacting against the foreign antigens of its new host (*13*).

A nuclear sex difference in rabbit heterophils was first observed by Lüers (*14*). Used as a biological cell label it provides a means of analyzing the causes of delayed death among irradiated animals treated with homologous bone marrow.

Under the conditions of our experiment not all animals receiving bone marrow showed a successful marrow transplant and a number of these failures died early from infection, but some regenerated their own bone marrow and lived. This underlines the fallacy of tacitly assuming in similar experiments, where a cell "label" is not used, that all treated survivors have marrow transplants.

From our results it seems evident that some deaths among animals with successful marrow homotransplants are undoubtedly due to rejection of the transplant by the host's recovered antibody-producing system. This rejection may occur early or late and is not necessarily fatal to the animal as a whole. But this phenomenon accounts for relatively few of the late deaths, most of which appear to be due to some other cause.

The findings of diarrhea and rapid weight loss in our main group of delayed deaths (7 animals died at the 33rd to 40th day postirradiation) agree with the results obtained in mice by Congdon and Urso (*15*). These authors also noted at autopsy the marked atrophy of the lymphoid tissues, and they, as well as Denko (*16*), who also investigated this problem in mice, commented on the completeness of the bone marrow "regeneration." That this bone marrow is, at least in major part, derived from homotransplanted cells and is fully capable of initiating and sustaining a marked granulocytosis is shown by our present findings. It thus seems that destruction of the marrow transplant by a delayed immunological response of the host to the foreign cells is not the mechanism in this group of late deaths.

The possibility that the homotransplanted marrow produces cells capable of antibody production and that this antibody-producing system, by virtue of its foreign origin, is immunologically active against all the tissues of the host certainly cannot be excluded on the present evidence. Indeed, many of our findings resemble closely the recent observations made by Billingham and Brent (*17*) when they induced tolerance by intravenous injection of newborn mice with adult spleen cells. They found that with some strain combinations the recipients, even though they developed normally and were perfectly healthy for 1 or 2 weeks, would suddenly become emaciated and die within a few days with gross

involution of the lymph nodes and abnormal fibrotic spleens. These facts, as the authors suggest, seem best explained as a result of immunological reactions produced by the transplanted spleen cells against host tissue antigens.

A third possibility is that in some animals host regeneration or perhaps donor repopulation of the depleted lymphoid tissue is slow, or incomplete, and that this state of affairs is accompanied by poor antibody production and resultant increased susceptibility to infection. Certainly the immediate cause of death in all our cases was infection, generally a diffuse bilateral bronchopneumonia.

The group of animals that escaped all these troubles and was alive and well with functioning marrow homotransplants 32 weeks after the last dose of irradiation is also of great interest. Did the antibody-producing tissue of these animals regenerate from host sources? If so, the restored host system appears to have acquired an altered immunological specificity, perhaps in the nature of an induced immunological tolerance (*18*) for donor marrow antigens, as is suggested by Main and Prehn (*19*). Alternatively, if the host's antibody-producing system was completely replaced by a comparable system derived from marrow donor cells then the reverse may have been accomplished—an induced immunological tolerance for tissue antigens of host origin.

When rabbits are used for X-irradiation studies certain special problems arise. First, the rabbit is unusual in that, unlike most other species, death from an initial severe reaction is frequent immediately after X irradiation. About 25 percent of rabbits exposed to a single whole-body dose of 1,000 r die within 24 hours in a state of shock; furthermore, males are more susceptible than females. However, after 500 or 600 r the initial mortality is slight, and rabbits weighing 2 kg., or more, are less sensitive than smaller animals (*20*).

It is also known that following 600 r total-body X irradiation in this species, antibody production is virtually abolished (*21*), and while a particular X-ray dose is generally less effective when it is given in fractions than when it is delivered at one time, there is even a suggestion that divided doses of irradiation might be more effective than single doses for lowering host resistance (*22*). Lamarque and Gary-Bobo (*23*), in a study of whole-body irradiation fractionated into two doses, have shown that in the guinea pig, whereas the LD50 is 250 r for a single dose, the LD50 for two equal doses, spaced 24 hours apart, is 355 r.

In the present study with these data in mind, it was decided to use 2.5 to 3.0 kg. animals and divided X-ray doses. In fact, this combination reduced the initial mortality to negligible proportions and, judging from the percentage of successful marrow transplants, retained about the same initial depressive effect on the host's ability to reject grafts as occurs after a single dose of 1,000 r whole-body X irradiation.

Second, persistent gastric retention with ulceration and rupture become important causes of death in irradiated rabbits after 9 days. Fractionating the X-ray dose failed to reduce the incidence of these deaths, but a

beneficial effect followed the use of postirradiation antibiotic therapy.

Although not the prime aim of the experiments reported here, it is clear that tetracycline hydrochloride administration considerably lowers mortality in lethally irradiated rabbits. This is largely due to elimination of early deaths from bacteremia occurring in agranulocytic animals. The gradual onset of infections in varying numbers of survivors 1 to 3 weeks after the cessation of tetracycline treatment was a feature of all groups; in some animals these late infections proved fatal.

Summary

1) Male New Zealand white rabbits were exposed to 1,100 r whole-body X irradiation fractionated into two doses: an initial dose of 600 r was followed 24 hours later by an additional 500 r. Subsequently, they were injected intravenously with homologous bone marrow from a female donor and treated with tetracycline hydrochloride for 3 weeks.

2) In 65 percent of the animals, persistence and function of the marrow homotransplant occurred as shown by the appearance of female heterophils in the peripheral circulation. Of this group, 15.5 percent died early from gastric perforation; in 19 percent, the marrow transplant was ultimately rejected by the host; 27 percent died from infection at 33 to 40 days with the marrow transplant intact and still functioning; and 38.2 percent were alive and well with an intact marrow homotransplant at 32 weeks.

3) In 30 percent the marrow transplant was unsuccessful. Of this group, 16.8 percent died from gastric perforation, 41.6 percent died from infection at 13 to 25 days, and 41.6 percent regenerated their own bone marrow.

4) Tetracycline hydrochloride treatment reduced the cumulative mortality of the irradiated rabbits from 90 to 75 percent at 32 weeks. The addition of bone marrow caused a further reduction to 60 percent.

5) Fractionation of the total-body X irradiation reduced the occurrence of immediate postirradiation death in shock.

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PLATES

Vol. 20, No. 1, January 1958
447911—58——22

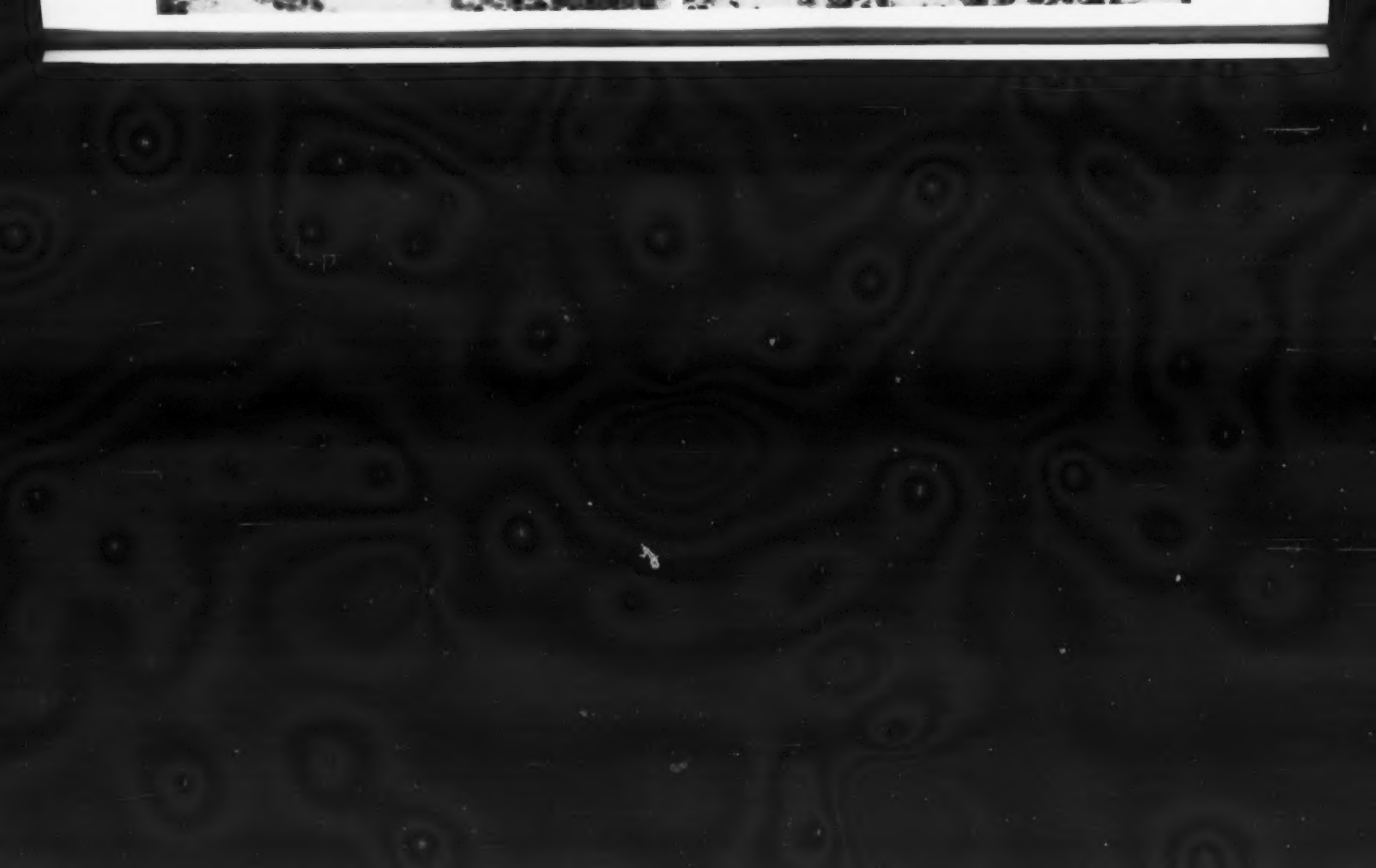
PLATE 16

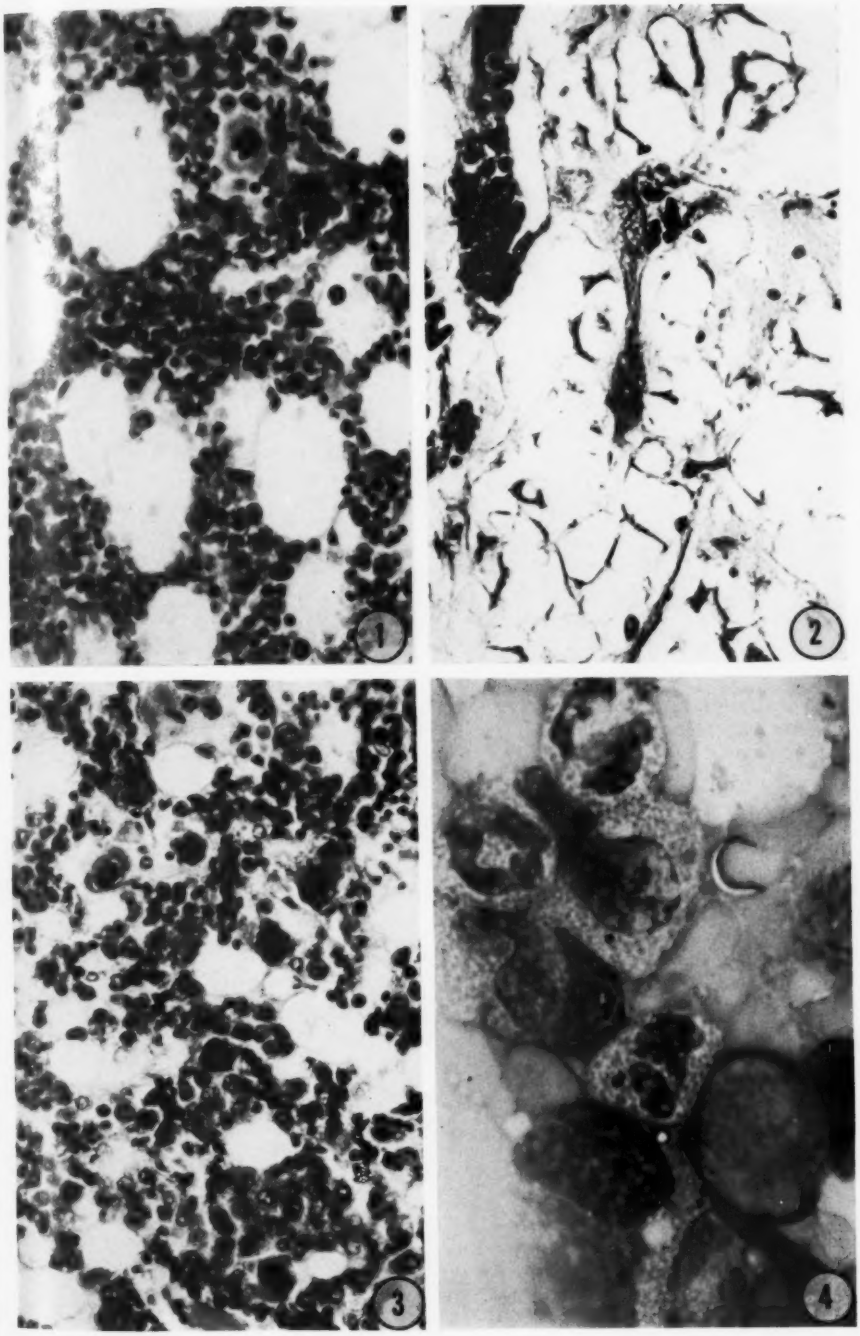
FIGURE 1.—Femoral bone marrow from normal male rabbit. Hematoxylin and eosin. × 400

FIGURE 2.—Marked destruction of femoral bone marrow in a rabbit that died 25 days after X irradiation and intravenous injection of homologous bone marrow. A successful marrow transplant has been rejected by the host. Hematoxylin and eosin. × 400

FIGURE 3.—Successful marrow transplant in a male rabbit that died with infection 36 days after X irradiation and intravenous injection of female bone marrow. Hematoxylin and eosin. × 400

FIGURE 4.—Marrow smear from same X-irradiated, marrow-treated male rabbit. A heterophil showing a nuclear "drumstick," characteristic of the female, can be seen. Wright's stain. × 1,550





Porter and Murray

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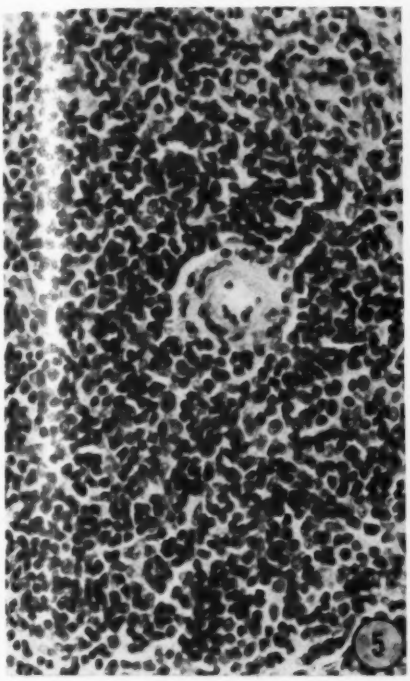
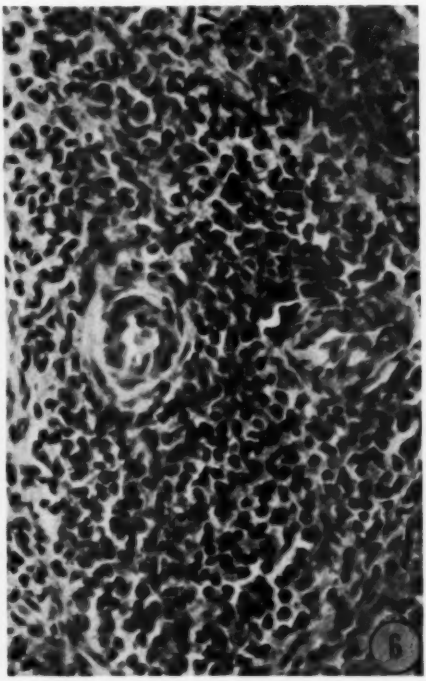
PLATE 17

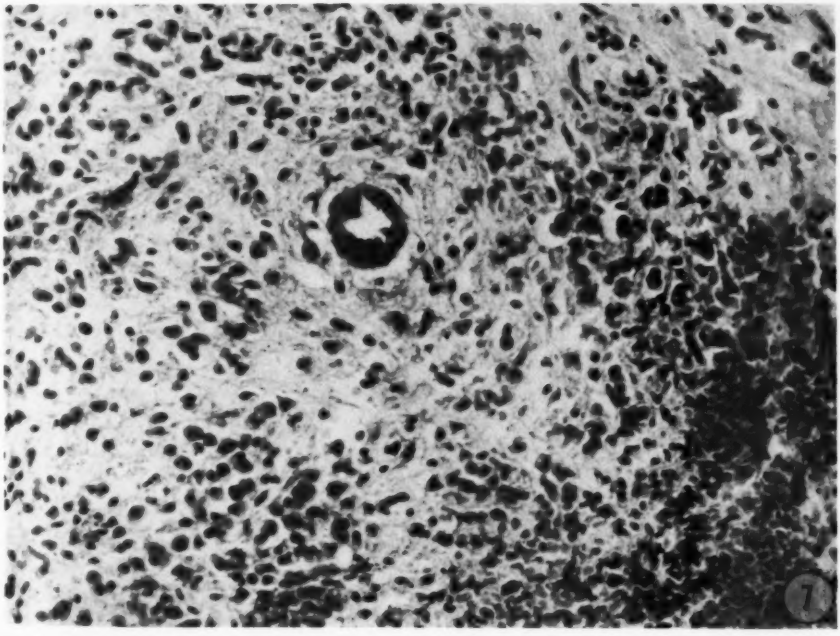
FIGURE 5.—Lymphoid nodule in spleen of normal male rabbit. Hematoxylin and eosin. × 400

FIGURE 6.—Advanced regeneration of lymphoid nodule in spleen of the X-irradiated, marrow-treated rabbit whose bone marrow is shown in figure 2. This animal died at 25 days after rejecting a marrow transplant. Hematoxylin and eosin. × 400

FIGURE 7.—Total destruction of lymphocytes in splenic lymphoid nodule of X-irradiated, marrow-treated rabbit whose bone marrow is shown in figures 3 and 4. This animal died with infection despite a successful marrow transplant. Groups of plasma cells can be seen and, in *lower right* of photograph, an area of hemorrhage and necrosis with some nuclear debris. Hematoxylin and eosin. × 400







Number of Mouse Histocompatibility Genes Involved in Skin Grafting from Strain BALB/cAn to Strain DBA/2 [1]

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The genetic theory of homograft resistance was evolved as the result of experiments in which tumor grafts, because of their convenience, were used as the test material. Very little work concerning histocompatibility genes (other than genes of human blood groups) has been performed with normal tissues. From what has been accomplished, it is apparent that both tumor and normal tissues obey the same general "Laws of Transplantation" (*1*). However, it is also well known that homografted tumor tissue is often capable of growth in a genetic system that would entirely suppress the growth of a normal tissue graft. This property has been ascribed to a "loss" of histocompatibility factors on the part of the tumor or to the ability of tumor tissue, because of its greater growth potential, to survive despite certain histocompatibility factors. Whatever the reason, it seems probable that tumor tissue tested in resistant backcross mice or in F_2-generation hybrids may give a lower figure for the number of histocompatibility factors than that expected for normal tissue. Hence, tumor tissue is an unreliable tool for measuring the number of histocompatibility loci operating when normal tissue homografts are made from one inbred strain to another.

Because much of the work in this laboratory involved the use of homografts taken from donor mice of strain BALB/cAn and transplanted to strain DBA/2 recipients, it became of particular as well as of general interest to obtain some idea of the number of histocompatibility loci operating in this specific system (*2–4*). Since Snell had already shown that the powerful *H-2* locus was not involved, it was possible that a close histocompatibility relationship existed between these strains (*1*).

Methods

The method of ascertaining the number of histocompatibility loci and the genetic theory involved has been discussed in detail (*5*). The method depends upon the fact that segregation of traits will occur in

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either the F_2 hybrid cross between the donor and recipient strains or in the progeny of the F_1 hybrids and mice of the recipient strain (resistant backcross). The number of loci can be calculated from the number of graft "takes" in mice of these 2 types.

In current work, 2 cm.^{2} full-thickness skin grafts rather than tumor grafts were used as challenge tissue. The skin-grafting technique has been previously described (*6*). The skin donors were strain BALB/cAn male mice, 3 to 6 months old; recipients were (BALB/cAn × DBA/2)F_2 hybrids and (BALB/cAn × DBA/2) × DBA/2 backcross mice, 2 to 3 months old, of both sexes. Previous work suggested that there were no sex-linked histocompatibility loci involved in this particular genetic system (*7*).

The end point in this study, as in the previous work from this laboratory (*3*), was the complete and entire sloughing of the graft, and not the loss of viability. A graft was recorded as "good" when the fur was luxuriant and there was little or no shrinkage, and was "poor" when there was shrinkage and/or little hair growth.

Results

The resistant backcross group of recipients was composed of 99 animals whose coat colors were: 29 cinnamon (*Dd bb Aa*), 26 chocolate (*Dd bb aa*), 20 dilute brown (*dd bb aa*), and 24 dilute-brown agouti (*dd bb Aa*). In this backcross group, 59 were female and 40 were male. Within 20 days after surgery, grafts had sloughed in 71 of the animals, and of the remaining 28, 18 were recorded as "poor." At 60 days after grafting, grafts had completely sloughed in all mice, except 3, and the remaining 3 grafts were so "poor" that eventual slough was regarded as inevitable and this part of the experiment was terminated. There was no observable correlation between coat-color and length of graft survival.

The F_2 hybrid group of test animals was composed of 60 male and 60 female mice. At 60 days after grafting, 10 of these mice still retained their grafts, but only 5 were considered "good." By 120 days all grafts except 4 had completely sloughed. After 200 days the experiment was terminated; at that time 3 grafts persisted and were recorded as "good."

Previous experience, part of which was published, indicated that BALB/cAn grafts to homozygous DBA/2 recipients were rapidly and uniformly unsuccessful (*3*).

Discussion

The failure of all grafts in the backcross group of 99 mice indicated that the number of histocompatibility loci involved probably exceeded 6 and that determination of the actual number by this method would require many more animals.

The survival of 3 grafts among the 120 F_2 mice, for 6 months at least, suggests that the number of histocompatibility loci was about 13. The expectation from genetic theory is 2.3 survivals for 13 loci among 100 F_2 animals. The result obtained is similar to that found by Eichwald in another genetic system (*8*).

The actual number of loci contributing to resistance might have been many times higher than 13, because, judging by the prolonged survival of some of the grafts, it is possible for a skin graft to survive despite the action of "weak" loci. The authors venture to suggest that the number of apparent loci would have been considerably higher if the test mice had been immunized against BALB/cAn tissue prior to challenge with the skin grafts.

If this line of reasoning is carried further, it may be argued that almost every locus at which the 2 strains differed may have served to some extent as a histocompatibility locus, although most of them were very weak, and that graft resistance was a threshold effect due to their combined action. In this event, the count of histocompatibility loci becomes rather meaningless, because the result is completely dependent upon the sensitivity of the method used. The growth of grafts in F_2 mice remains a practical measure of the degree of *foreignness* even if it is not an accurate expression of the number of genes involved. The results obtained indicate that the 2 strains were not similar in respect to histocompatibility factors.

Summary

The number of histocompatibility loci operating when skin grafts are made from strain BALB/cAn donors to strain DBA/2 recipients was estimated by challenging F_2 and resistant backcross mice with BALB/cAn skin grafts. The survival, for at least 6 months, of 3 grafts among 120 F_2 mice and no graft survivals among 99 backcross mice indicated that the number of apparent loci was approximately 13. The authors suggest that homograft resistance may be a threshold effect due to large numbers of relatively weak loci, which invalidates actual counts of these loci. It is concluded that the 2 strains used were not very similar so far as histocompatibility was concerned and that graft survival in the F_2 hybrid was a valuable measure of the degree of *foreignness* between the strains, although not an accurate measure of the number of genes involved.

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The Influence of Amethopterin on the Survival Time of Leukemic Mice with Respect to Food Intake, Body Weight Changes, and Tumor Growth [1]

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It has long been recognized that simple caloric restriction may result in the retardation of growth of both the transplantable tumor and its host (*1–8*). In an investigation of the selectivity of antineoplastic chemotherapeutic agents it is therefore desirable to determine to what extent unspecific host toxicity, as reflected in reduction in food intake and weight loss of the host, may account for the observable antitumor effects. Goldin *et al.* (*9*) demonstrated that the growth of sarcoma 180 is correlated with reduced food intake and body weight loss resulting from drug administration. Most of the tumor retardation observed with various nitrogen mustards could be accounted for on the basis of reduced food intake and body weight loss resulting from drug toxicity. Bennette (*10*) has presented evidence that the antineoplastic action of amethopterin for sarcoma 180 and sarcoma 37 is mediated primarily through unselective toxicity of the drug for the tumorous host. Retardation of these tumors occurred to an important extent only when dosages were employed which produced marked toxicity, as reflected in reduction in food intake, body weight loss, and the death of individual mice. Other investigators have also noted the correlation of host toxicity and antitumor effect for folic acid analogues, but have considered that the extent of toxicity for the host was not sufficient to account for the antitumor effect (*11–13*).

Treatment with amethopterin, whether initiated early or late against transplantable leukemia, can produce marked increases in the survival time of the mice (*14, 15*). The current study was conducted to determine:

(*a*) The effect of amethopterin, on the food intake, body weight, and tumor growth in leukemic mice at doses that increase survival time.

(*b*) The extent to which the increase in survival time of leukemic mice treated with amethopterin can be duplicated by simple caloric restriction.

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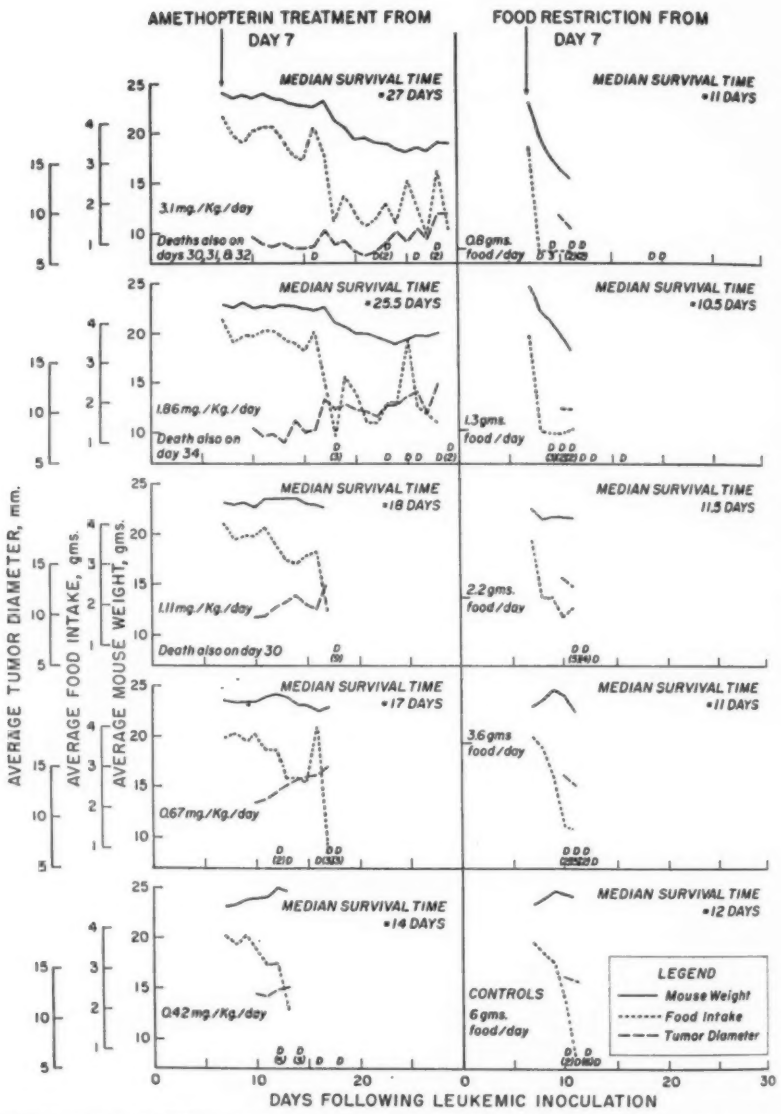


TEXT-FIGURE 1.—Effects of amethopterin treatment and of food restriction on animal weight, daily food intake, tumor size, and survival time of mice with advanced leukemia. Daily treatment with amethopterin and food restriction were initiated 7 days following leukemic inoculation. Averages were obtained for surviving animals and are shown only when based on three or more mice. Days of death of individual mice are indicated by the symbol D with figures in parentheses showing multiple deaths occurring in a group on a day. Other groups of tumor-bearing mice in the experiment, but for which data are not shown above, include: food restriction levels of 0.5, 0.3, and 0.2 gm. daily; a completely starved group; and a group maintained on a diet isocaloric with that consumed by the 0.67 mg. per

Methods

General.—Eight- and 12-week-old hybrid male mice (BALB/cAn × DBA/2J)F_1 were employed in experiments 1 and 2, respectively. The animals were inoculated in the right hind leg with 0.1 ml. of a saline suspension of leukemic (L1210) cells. Tumor cell suspensions were prepared from the local tumor growth (experiment 1) or from the spleen tissue (experiment 2) of stock (DBA/2J) tumor-bearing mice. Inoculation of serially diluted leukemic cell suspensions in normal mice showed that the concentration of cells in the suspension employed in each experiment was at least 100 times the concentration required to elicit death from tumor growth in 100 percent of untreated mice.

Amethopterin was dissolved in two percent aqueous sodium bicarbonate and injected daily, either intraperitoneally (experiment 1) or subcutaneously (experiment 2), in the constant volume of 0.01 ml. per gm. of body weight.

The various groups of both experiments contained 10 mice each, and the mice were housed in individual cages. Each mouse scheduled to receive food *ad libitum* was offered 6 gm. of unpelleted Purina laboratory chow per day. Proportionally less food was offered to animals whose diets were to be restricted. All mice received water *ad libitum*. Details of procedures employed in experiments involving the controlled feeding of mice have been previously reported (*9*, *16*).

The diameter of the local tumor at the site of leukemic inoculation was estimated by manual palpation of the tumor and compared with spherical beads of known diameters. Daily determinations were made of the weight of the mice, food intake, tumor dimensions, and survival time.

Results

The principal results of the two experiments are given in text-figures 1 and 2.

Untreated controls on an *ad libitum* diet showed median-survival times of 12 days and 11.5 days, respectively. Terminally, the local tumor at the site of the leukemic inoculation had increased in size and the dietary intake dropped abruptly. Deaths apparently occurred too soon for the reduction in food intake to be reflected in loss of animal weight.

In the first experiment (text-fig. 1) a comparison was made of amethopterin treatment and food restriction, both begun 7 days after leukemic inoculation. The longest median-survival time (27 days) was achieved

TEXT-FIGURE 1.—Continued

kg. amethopterin group. All the food-restricted groups and the starved group dropped in weight rapidly and died with a median-survival time of 10.5 or 11 days, the average tumor diameter being generally about 10 mm. on day 10. The isocaloric group did not lose weight but still died early, the median-survival time being 11 days; on day 10 the average tumor diameter was 14 mm. Over the period of the experiment, a group of non-tumor-bearing controls maintained an average daily food intake of about 4 gm. and gained about 4 gm. in weight between day 7 and 30.

with the highest dose of amethopterin employed (3.1 mg. per kg. daily). The food intake of this group was maintained at high levels for a sustained period, during which there was no appreciable weight loss of the animals. In addition, tumor growth was inhibited over an extended period. Eventually, however, tumors did begin to show progressive growth, food intake was reduced, and loss in weight became more evident.

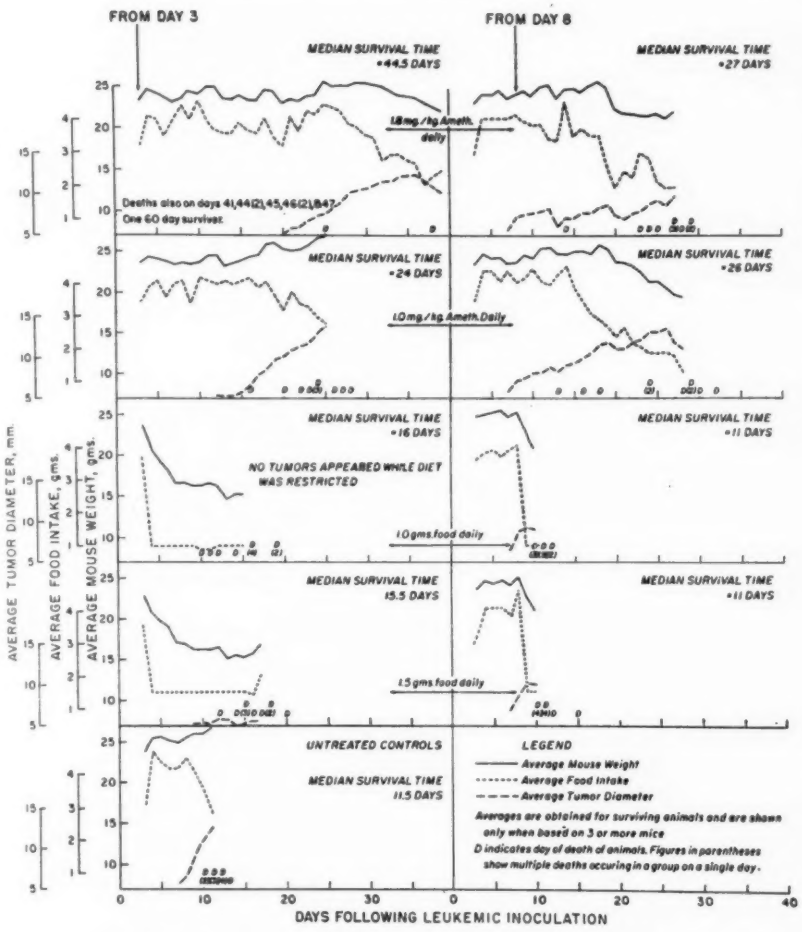
As the dose of amethopterin was reduced, progressive tumor growth, diminished food intake, and death occurred earlier. At the lower amethopterin levels, where increases in survival time were not extensive, the reductions in dietary intake were not reflected in the weights of the mice prior to death.

None of the restricted diets increased the median-survival time of the mice. With moderate restrictions in food available, the results paralleled those for the control mice: Food intake dropped below the amount of food available, deaths occurred early accompanied by no serious animal weight loss, and the tumors were large at the time of first palpation (10 days). When food was severely restricted, the mice ate all available food, lost weight, and in the main, showed no increase in survival time. At the time of the first tumor palpation (10 days) these mice tended to have smaller tumors. The survival times of several such mice were prolonged, but this was accompanied by severe weight loss (*e. g.*, at 0.8 gm. per day, 2 mice survived to the nineteenth and twentieth day following tumor implantation, respectively, and died with small tumors; the mouse weights were reduced to 15 gm. and 13 gm. one day prior to death). During the course of this experiment, non-tumor-bearing controls (data not shown) showed progressive weight gain; the average food consumption fluctuated in the neighborhood of 4 gm. per day.

In the second experiment (text-fig. 2), a comparison was made of amethopterin treatment and food restriction, beginning on the third and eighth day after leukemic inoculation. The results for both amethopterin treatment and dietary restriction beginning on the eighth day closely paralleled those obtained in the preceding experiment in which treatment or dietary restriction was initiated on the seventh day. Treatment with amethopterin at appropriate levels resulted in a retardation of tumor growth and an extension of survival time, animal weight and food intake initially being maintained and then diminishing. Dietary restriction did not yield any definite extensions in survival time although there was some indication that tumor growth had been retarded.

The results for amethopterin treatment, started on the third day, differed from that begun on the eighth day principally in degree. At 1.8 mg. per kg. of amethopterin daily, no definite tumors appeared until the twentieth day, but thereafter the tumors grew progressively. The median-survival time was increased to 44.5 days in contrast to 27 days for the later initiation of treatment. Food intake and body weight were maintained for an extended period of time.

In contrast to the observations with advanced leukemia, dietary restriction begun early was not only successful in retarding tumor growth,



TEXT-FIGURE 2.—Effects of amethopterin treatment and of food restriction in early and advanced leukemia. Daily treatment with amethopterin and food restriction were initiated on the 3rd and the 8th day following leukemic inoculation. Drug treatment and food restriction were discontinued for a group following the death of 60 percent of the animals in the group. Data are not shown for groups restricted to dietary levels of 0.5 gm. of food per day—this level of food intake inhibited tumor growth but also produced extreme weight loss and early death of mice. The median-survival time was 8.5 days when food restriction was begun on day 3, 11.5 days when restriction was begun on day 8.

but also achieved definite increases in survival time. Mice on a restricted diet of 1.5 gm. of food daily (about 40 percent of the normal mouse daily intake) showed an increase in median-survival time from 11.5 days to 15.5 days and their tumors showed little if any progressive growth. At 1.0 gm. of food daily (about 25 percent of the normal daily food intake)

the median survival time was increased to 16 days and no definite tumors appeared among these mice during the period of dietary restriction. In both of these restricted diet groups, mice lost about one third of their initial average weight.

Discussion

The present experiments show that the antileukemic action of amethopterin is not dependent upon any reduction in food intake or animal weight loss that it may produce. In neither one of the experiments did restriction in food intake accomplish the increases in survival time or antitumor effects obtained with amethopterin treatment. Further, treatment with doses of amethopterin effective in increasing survival time served to maintain for a considerable period of time, rather than diminish, the dietary intake of the tumor-bearing mice.

The host-tumor-drug relationships are apparently as follows: The growth and infiltration of the leukemic cells is sufficient, in advanced stages of the disease, to restrict food intake sharply. This reduction in food intake may be not only symptomatic, but also a contributing factor to death. Evidence has been presented by Mider *et al.* (*17*) that the tumor "robs" the host of essential metabolites. Food intake is not sufficient to meet the requirements of both the tumor and the host so that the tumor grows at the expense of the host (*17–19*). With progressive tumor growth, reduction in food intake has been observed to be accompanied by reduction in carcass weight (*16, 17*). Under these circumstances the terminal reduction in food intake could become critical to the survival of the host.

The competitive nature of the host-tumor relationship is illustrated further by the following observations:

(*a*) Feeding of a desiccated tumor preparation resulted in an increase in carcass weight and tumor weight in rats with Walker carcinosarcoma 256 (*20*).

(*b*) Older, heavier mice with leukemia (L1210) or sarcoma 37 lived longer than younger, lighter mice (*16, 21*).

Amethopterin treatment alone can produce moderate or sharp reductions in food intake, in accordance with the level and frequency of treatment (*10, 22*). At the relatively low treatment levels employed in these experiments, amethopterin produced, in the initial period of treatment, little or no reduction in food intake. Apparently, by inhibiting the growth of the leukemic cells, the drug not only served to prevent the sharp reduction in dietary intake that such tumor growth would produce but also tended to diminish the critical nature of the food requirement by the host. For example, mice treated with amethopterin showed marked increases in survival time despite the fact that the food intake was eventually reduced for an extended period. Nevertheless, the importance of prolonged maintenance of food intake, even where treatment is being effective, is supported by the preliminary observation (*22*) that when treatment is initiated after the food intake has been reduced, the mice that live the longest are those

in which the treatment has restored the food intake closest to normal levels.

The eventual weight loss of the host and reduced food intake are presumably due to the combined effects on the host of tumor growth and cumulative amethopterin toxicity.

Further evidence that the antileukemic action of amethopterin is not attributable to nonspecific host toxicity is provided by auxiliary hematological observations, made in connection with the first experiment, which paralleled those for food intake (*23*). Ordinarily, leukemic growth results in a terminal rise in peripheral-blood leukocyte count and fall in hemoglobin concentration. Effective treatment with amethopterin resulted in longer maintenance at normal levels of peripheral-blood leukocytes and hemoglobin concentration, even when treatment was initiated late in the course of the disease.

Although dietary restriction resulted in minimal effect against advanced leukemia, it is evident from the second experiment that dietary restriction initiated early in the course of the disease, although accompanied by marked weight loss, produced definite retardation of tumor growth and prolongation of life. Apparently when the leukemic cells are still relatively low in number, the host can tolerate the degree of underfeeding required to elicit antileukemic action. This effect of dietary restriction on tumor volume and survival time of mice with early leukemia would suggest that, when drug treatments are applied early, extreme caution should be used in employing reduction in tumor volume or increase in survival time as indices of the effectiveness of chemotherapy.

Summary

Two experiments were conducted comparing the effectiveness of amethopterin administration and of dietary restriction in the treatment of the transplanted mouse leukemia L1210. Treatment with amethopterin tended to maintain, rather than diminish, the food intake of the tumor-bearing animals, indicating that the antitumor effectiveness of such treatment was not dependent on any reduction in food intake that the drug might produce.

Although dietary restriction initiated late in the course of the disease resulted in some retardation of local tumor growth and weight loss of the host, except for individual instances, it did not increase survival time. When dietary restriction was initiated early, animal weight loss was accompanied by retardation of tumor growth and definite increases in survival time.

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Effects of Cortisone and Adrenalectomy on the Growth Rate of Ehrlich Ascites Tumor in Mice [1, 2, 3]

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Many workers in recent years have studied the effects of cortisone (17-hydroxy-11-dehydrocorticosterone) and other adrenocortical hormones on sarcomas and carcinomas in man and animals (*1–4*). In general, cortisone appears to depress neoplastic growth, though it is difficult to assess most of its effects in quantitative terms. An increased tumor growth rate in adrenalectomized animals has been reported (*5–8*). However, relatively little work in this connection has been done with fluid tumors, which are desirable for quantitative studies (*9, 10*), because the total number of tumor cells can be determined simply and accurately. For this reason, the Ehrlich ascites tumor has been chosen for studying the effect of varying dosages of cortisone and the effect of adrenalectomy on the growth rate of the tumor in the experiments reported here.

Materials and Methods

Female Swiss mice, obtained from a commercial mouse farm and weighing 18 to 25 gm., were used in most of the experiments. Twenty-two strain CFW female animals, purchased from another source, were used as controls in group II, but the growth rate of these showed no statistically significant difference from the Swiss strain. The drinking fluid was tap water, unless otherwise noted. The solid diet, which was a standard block-type laboratory chow, and the drinking fluid were available to the animals at all times.

The data presented are based on seven groups of experiments. Ehrlich ascites-tumor fluid was obtained from host mice in the laboratory of Dr. Ralph N. Baillif. The fluid was drawn from the host under antiseptic conditions and a sample was taken to determine the number of cells per ml. of fluid. A tumor-cell count was obtained on the fluid from each mouse by diluting a pooled sample with white cell diluting fluid in a red cell pipette.

[1] Received for publication September 13, 1957.

[2] Part of a dissertation submitted to the Department of Anatomy of the Graduate School of Tulane University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] This investigation was supported by research grant C-1563 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and in part by the American Cancer Society, Louisiana Division.

[4] The author wishes to express his sincere appreciation to Dr. Ralph N. Baillif and Dr. Huldah Bancroft, for their valuable assistance.

The count was made in a Neubauer hemacytometer, and calculated as for a white cell count. From this count, the volume necessary to provide the requisite dose of 11.0 to 13.0 $\times$ 10^6 cells was ascertained. A number of cells within this range was given each mouse.

In order to withdraw the accumulated ascitic fluid, the skin of the abdomen was cut along the median line and pulled to the side; the muscular layer was lifted with forceps, and a small incision made into the cavity. The ascitic fluid was withdrawn quantitatively and the total volume from each animal recorded. The total volume of fluid multiplied by the cell count per ml. gave the total number of cells present in the peritoneal cavity of each mouse. Although the rate of growth of tumor cells has been shown to be logarithmic in character, no determination of the particular character of the growth could be obtained in this experiment. Therefore, an estimate of the rate of growth was made by the following method: The number of cells given in the initial inoculation was subtracted from the total cell count to give the number of cells produced during the incubation period. This number was divided by the number of days of incubation to give a figure representing the cell increase per day.

The daily tumor-cell increase was obtained by dividing the cell increase per day by the number of cells originally injected. This percentage increase is the figure used to compare the tumor growth rates of the animals in the various experiments. Except for the long-term incubation periods in group IV, all animals were sacrificed on the tenth day by means of chloroform.

The average daily tumor-cell increase for each group of experimental animals was compared with that of other groups and the probability that the groups were different was determined by the t test. The following formula was used to find the t value:

Subscript 1 designates one group of animals and *subscript 2* designates the other groups compared.

x = Daily tumor-cell increase of an individual;

$\bar{x}$ = average daily tumor-cell increase for a group;

n = number of individuals in the group.

$$\bar{X}_1 = \frac{\sum x_1}{n_1} \qquad \bar{X}_2 = \frac{\sum x_2}{n_2}$$

$$S_1^2 = \frac{n_1 \sum x_1^2 - (\sum x_1)^2}{n_1(n_1-1)}$$

$$S_2^2 = \frac{n_2 \sum x_2^2 - (\sum x_2)^2}{n_2(n_2-1)}$$

$$S^2 = \frac{S_1^2(n_1-1) + S_2^2(n_2-1)}{n_1+n_2-2}$$

$$t_{(n_1+n_2-2)} = \frac{\bar{x}_1 - \bar{x}_2}{S}\sqrt{\frac{n_1 n_2}{n_1+n_2}}$$

Animals used in these experiments were placed in seven categories.

Group I.—A known number of cells of the Ehrlich ascites tumor was given asceptically to three series totaling 49 mice. Each mouse received 5.0 mg. of cortisone[4] in the muscles of the thigh on alternate days beginning with the day of tumor inoculation and continuing until a total of four injections (20.0 mg.) had been completed.

Group II.—A known number of tumor cells was injected into the peritoneal cavity of each of 41 mice. Twenty-two of these mice were strain CFW. Comparison of these animals with the Swiss controls showed that the daily tumor-cell increase was not significantly different in the two strains. Fifteen control animals were given an amount of saline equivalent to the cortisone dosage of those in group I. The daily tumor-cell increase of these animals and those controls that did not receive saline injections did not differ significantly.

Group III.—Intramuscular doses of cortisone, 2.5 mg., were given to each of 39 mice on alternate days until a total of 10 mg. had been administered. This is half the dose of cortisone given the animals in group I. On the day of the first cortisone injection each mouse was inoculated, intraperitoneally, with 10 to 12 million tumor cells. All members of this group were killed the tenth day after tumor injection.

Group IV.—These experimental animals were given a known number of tumor cells and four 5.0 mg. injections of cortisone intramuscularly. The first injection of cortisone was made on the day of tumor inoculation and injections were repeated on alternate days until a total of 20.0 mg. of the drug had been administered. It was originally planned to kill these mice on the twentieth day after inoculation, but none survived the entire period. Data were obtained on 14 animals by drawing off the fluid just at death, or when death appeared imminent.

Group V.—Bilateral adrenalectomy was done on 48 mice. Each mouse was anesthetized by injecting a solution of approximately 1.8 mg. of sodium pentobarbitol (Nembutal®) in 0.09 ml. distilled water into the peritoneal cavity. The skin was incised along the dorsal midline and pulled to either side as needed. Lateral to the dorsal longitudinal muscles on either side, an incision was made and small gauze sponges were placed in the abdominal cavity to retract the viscera from the adrenal glands and to absorb any fluid present. The adrenals were excised, sponges removed, and the incisions sutured with surgical cotton. The skin closure was protected with a film of collodion. These mice were placed in a recovery cage for the first 7 to 10 postoperative days and were given drinking water containing 12.5 mg. Terramycin® per liter in 0.9 percent saline. It has been shown that saline (*11*) and Terramycin (*12*) in drinking water increase the survival rate of adrenalectomized animals. After sufficient time had elapsed for recovery from the operation, usually 7 to 10 days, each animal was given a known number of ascites cells. These cells were allowed to grow in the peritoneal cavity for 10 days, at which time the mice were sacrificed.

[4] The cortisone (Cortone®) used in these experiments was supplied through the courtesy of the Merck & Co., Inc.

TABLE 1.—*Data on tumor growth rates*

Group	Series	No. mice	Description	No. cells injected $\times 10^6$	Days postinoculation at sacrifice	Cell increase per day (*mean* ± *S.D.*)
I	1	11	Cortisone, 5.0 mg., on alternate days; total of 20 mg.	11. 60	10	
	2	19		13. 50	10	
	3	19		9. 95	10	
Total		49				0. 48 ± 0. 46
II	1	8	Controls	14. 0	10	
	2	5		13. 5	10	
	3	28		11. 6	10	
Total		41				4. 94 ± 2. 96
III	1	16	Cortisone, 2.5 mg., on alternate days; total of 10 mg.	10. 9	10	
	2	23		12. 4	10	
Total		39				1. 22 ± 0. 82
IV	1	14	Cortisone, 5.0 mg., on alternate days; total of 20 mg.	11. 9	11 to 15	0. 81 ± 0. 99
V	1	6	Adrenalectomized; drinking fluid with Terramycin and saline.	12. 36	10	
	2	7		11. 57	10	
	3	8		12. 67	10	
	4	12		12. 30	10	
	5	6		12. 50	10	
	6	9		12. 69	10	
Total		48				7. 87 ± 4. 11
VI	1	16	Sham operated; drinking fluid with Terramycin and saline.	13. 14	10	
	2	8		12. 95	10	
Total		24				4. 95 ± 5. 81
VII	1	14	Drinking fluid with Terramycin and saline.	13. 15	10	
	2	13		12. 36	10	
Total		27				6. 56 ± 3. 14

Group VI.—Animals used as controls for the adrenalectomized group were sham operated. The operative procedure used in group V was duplicated in this group of 24 mice except that the adrenal glands were not excised. The drinking water was the same as that given the adrenalectomized group. These animals were sacrificed 10 days after tumor inoculation and the tumor-cell growth rate was determined.

Group VII.—Twenty-seven mice were given Terramycin, 12.5 mg. per l., in 0.9 percent saline as drinking water for 7 days before tumor inoculation and thereafter until sacrifice. These animals were not sham operated.

Results

Group I.—An average daily tumor-cell increase of 0.48 was obtained for this group of 49 cortisonized animals. Goldie *et al.* (*9*) found a similar depression of tumor growth of sarcoma 180 in its ascitic form when mice were injected with cortisone. The data for all groups of experimental mice are presented in table 1.

Group II.—Forty-one control animals were given tumor cells intraperitoneally and were sacrificed on the tenth day after inoculation. The average daily tumor-cell increase was 4.94.

Group III.—The 39 animals in this group received 2.5 mg. doses of cortisone on alternate days until a total of 10 mg. had been administered. The average daily tumor-cell increase was 1.22. This was significantly higher than the growth rate of animals receiving the larger dosage and was significantly lower than the tumor growth rate in controls.

Group IV.—The mice in this group were allowed to remain alive as long as possible in order to determine the long-term effect of cortisone on the tumor growth rate. Fourteen animals survived 11 to 15 days and were studied in this series. The average daily tumor-cell increase was 0.81.

Group V.—In this group of bilaterally adrenalectomized mice the average daily cell increase of the tumor was 7.87.

Group VI.—In this group of 24 sham-operated control animals the average daily cell increase was 4.95. This is a significantly lower rate than was obtained in the adrenalectomized group.

Group VII.—The animals in this group were given Terramycin in saline for drinking water, but were not sham operated, and served as a control group for groups V and VI. The daily tumor-cell increase of these animals was 6.56.

The daily tumor-cell increases in the seven groups of mice were compared statistically using the formula given under "Materials and Methods." The results of these comparisons are shown in table 2.

TABLE 2.—*Statistical comparisons of growth rates*

Comparison	Probability of similarity (t table)
Group I with group II	0. 001
Group III with group II	0. 001
Group I with group III	0. 001
Group IV with group I	0. 4
Group IV with group III	0. 2
Group V with group VI	0. 001

Gross and microscopic examinations of adrenal gland, thymus, lymph node, liver, and spleen from the groups that received cortisone showed changes similar to those reported by other workers (*13–15*). The number of lymphocytes was reduced in organs ordinarily containing these cells. Changes in the adrenals were especially notable in the zona reticularis where the cells appeared smaller and in the sinusoids which were increased in width.

Discussion

A comparison of the daily tumor-cell increase of group I mice, which received 5.0 mg. cortisone injections, with group II mice, which were controls, shows a statistically significant difference between the two groups (table 2). The cell increase of the tumor was 0.48 in group I and 4.94 in group II (table 1). This indicates that cortisone dosages of 5.0 mg. on alternate days (total of 20 mg.) have a definitely inhibiting effect on growth of the Ehrlich ascites tumor.

There is also a significant difference between the daily cell increase of the tumor in the controls of group II and in mice of group III that received 2.5 mg. cortisone on alternate days (total of 10 mg.) (table 2). The daily cell increase of tumors in mice of group I was compared with group III and, again, these two groups differed significantly (table 2); the animals that received the larger dosage of cortisone having the lower tumor growth rate. It is evident from these comparisons that the daily tumor-cell increase is depressed by 5.0 or 2.5 mg. cortisone on alternate days and that the larger of these doses depresses the rate to a greater extent than does the smaller dose.

Data from 14 animals in group IV gave a daily tumor-cell increase of 0.81 (table 1). Comparisons of this group with groups I and III (table 2) showed perhaps a significant difference. These data indicated that the inhibiting effects of cortisone diminish after administration is ended. There is apparently no long-term effect of the drug on tumor growth, but it would be unwise to draw a definite conclusion on the basis of the available data.

There is a significant difference between the daily tumor-cell increase of group VI (sham operated) and group V (adrenalectomized), as indicated in table 2. The daily increase of 7.87 for adrenalectomized animals is greater than that of 4.95 in sham-operated animals. The value of 6.56, as the daily cell increase of tumor in animals treated as the adrenalectomized and sham-operated animals but neither adrenalectomized nor sham operated, suggests that the operation introduces a stress in the mice that somehow lowers the tumor growth rate. Possibly the operative trauma acts as a stress mechanism activating an agent that inhibits the tumor when it is introduced.

Summary

Injections of 5.0 and 2.5 mg. of cortisone every other day for a total of four injections significantly inhibit the growth rate of the Ehrlich ascites tumor in strain CFW mice. The inhibition, within the limits of these

experiments, is proportional to the amount of cortisone injected. The growth rate of the tumor in animals receiving 5.0 mg. doses of cortisone was 0.48; in animals receiving 2.5 mg. doses, the rate was 1.22 compared with 4.94 in controls.

The growth rate of the Ehrlich tumor is significantly increased in animals that have been adrenalectomized, the rate being 7.87 as compared with 4.95 in sham-operated controls.

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Announcement

JOURNAL OF THE NATIONAL CANCER INSTITUTE

Sixteen-Year Cumulated Index

The National Cancer Institute has just issued a Cumulated Index of the Journal of the National Cancer Institute for the period 1940–56. Included are a subject index, with comprehensive cross references, an author index, and a list of current nomenclature for certain chemical compounds mentioned in the Index. This will be a valuable addition not only to those libraries that have files of the Journal, but also to research scientists in general, since it gives a rather comprehensive view of a large segment of cancer research during the 16-year period.

Copies of the Index have been sent to all libraries and individuals now receiving the Journal. Additional copies are available and may be had upon request to the Information Office, National Cancer Institute, T–19, Bethesda 14, Maryland.

Availability of Copies of Previous Issues

The National Cancer Institute has a few copies of some previous issues of the Journal and as far as the supply permits will be glad to fill requests from scientific libraries and investigators.

Subscriptions

The Journal of the National Cancer Institute is available on a subscription basis from the Superintendent of Documents, Government Printing Office, Washington 25, D. C. The subscription price for two volumes a year in the United States, Canada, and Mexico is $20.00; foreign $25.00. In appropriate cases the Journal is supplied without charge to scientific libraries.

SOCIETÀ ITALIANA DI CANCEROLOGIA

The First National Congress of the Italian Society of Cancerology will meet in Milan, Italy, on April 8 and 9, 1958. Professor Felice Perussia will preside.

The following subjects will be discussed:

1) Cancer of the mouse breast with reference to human mammary cancer.

2) Cancer of the esophagus.

For information apply to Dr. U. Veronesi, Secretary of the Congress, P.le Gorini 22, Milan, Italy.

228

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The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL 20 • NO. 1 • JANUARY 1958

Page

A comparison of impaired function with morphologic changes occurring in livers of rats ingesting *N*-2-fluorenyldiacetamide. *Harold P. Morris, Billie P. Wagner,* and *Louise S. Lombard* 1

Polycyclic aromatic hydrocarbons in coffee soots. *Masanori Kuratsune* and *W. C. Hueper* 37

Effects of 6-azauracil on galactose metabolism in mice, dogs, and man. *Allan H. Levy, Richard D. Fritz, Elizabeth P. Anderson,* and *Bernard R. Landau* 53

Observations of tryptophan staining of the pancreatic *alpha* cells. *Herbert J. Levine* and *George G. Glenner* 63

A method for the detection of *N*-formiminoglutamic acid in urine. *Milton Silverman, Rita C. Gardiner,* and *Paul T. Condit* 71

Cancer mortality among native white, foreign-born white, and nonwhite male residents of Ohio: Cancer of the lung, larynx, bladder, and central nervous system. *Thomas F. Mancuso* and *Elizabeth Jackson Coulter* 79

Epithelial tubule formation by mouse metanephrogenic mesenchyme transplanted *in vivo*. *Clifford Grobstein* and *George Parker* 107

Studies on the difference in sarcoma-producing capacity of two lines of mouse cells derived *in vitro* from one cell. *Katherine K. Sanford, Ruth M. Merwin, Gwendolyn L. Hobbs, Mary C. Fioramonti,* and *Wilton R. Earle* 121

Quantitative studies on Rous sarcoma virus. I. The titration of Rous sarcoma virus on the chorio-allantoic membrane of the chick embryo. *Alfred M. Prince* 147

A small-animal analytical respirometer. *Arnold W. Pratt* 161

Observations on the energy metabolism of rats receiving Walker tumor 256 transplants. *A. W. Pratt* and *F. K. Putney* 173

Long-term study of X-irradiated rabbits with bone-marrow homotransplants. *K. A. Porter* and *J. E. Murray* 189

Number of mouse histocompatibility genes involved in skin grafting from strain BALB/cAn to strain DBA/2. *Richmond T. Prehn* and *Joan M. Main* 207

The influence of amethopterin on the survival time of leukemic mice with respect to food intake, body weight changes and tumor growth. *Stewart R. Humphreys, Abraham Goldin, John M. Venditti,* and *Nathan Mantel* 211

Effects of cortisone and adrenalectomy on the growth rate of the Ehrlich ascites tumor in mice. *Ben E. M. Watson* 219

Announcements: Journal of the National Cancer Institute 227

Società Italiana di Cancerologia 228